

INFLUENZA VIRUS AND PROTEOLYTIC BACTERIA CO-INFECTION IN RESPIRATORY TRACT FROM INDIVIDUALS PRESENTING RESPIRATORY MANIFESTATIONS

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SUMMARY

A role for proteolytic bacteria in the exacerbation of influenza virus has been shown in natural hosts such as pigs and humans. Four hundred seven samples were collected from the respiratory tract of individuals presenting clinical manifestations, during influenza season (2003-2005) in São Paulo City. The aim of this study was to evaluate the incidence of determined bacteria co-infecting virus in human respiratory tract. Tests, such as bacteriological, immunofluorescence (IF), RT/PCR and hemagglutination (HA) were used for bacterial and viral investigation. Thirty seven (9.09%) positive for influenza virus were screened by IF. The RT/PCR confirmed the presence of influenza virus in these samples. Bacterial and agar casein tests demonstrated that 18 (48.64%) individuals were infected with proteolytic bacteria such as *Staphylococcus* spp., *Streptococcus* spp. and *Pseudomonas* spp. Among these samples, 13 (35.13%) were co-infected with influenza A virus. Influenza type B, co-infecting bacteria were found in five (13.51%) samples. *In vitro* the *S. aureus* protease increased the influenza HA titer after contact for 30 min at 25 °C. Results revealed the occurrence of co-infection with proteolytic bacteria and influenza in the evaluated individuals. This finding corroborates that virus versus bacteria synergism could be able to potentiate respiratory infection, increasing damage to hosts.

KEYWORDS: Influenza; Proteolytic bacteria; Co-infection; Respiratory tract.

INTRODUCTION

Virus-bacteria association^{3,17,18} infections has been reported to be one of the major causes of severe influenza pneumonia in human, possibly due to the synergistic effect of these microorganisms during the respiratory tract invasion. On the one hand, the influenza virus requires an enzymatic action to achieve the hemagglutinin molecule cleavage that increases its infectivity in host cells; this action is provided by the bacterial protease. On the other hand, the influenza virus can promote bacteria adherence on the host cells, where the bacterial adhesins are able to act when the cells are infected by this virus¹⁶.

The interaction between bacteria and influenza virus has been studied *in vitro* as well as *in vivo*, using different proteases such as urokinase, kallikrein, plasmin and thrombin in comparison with the bacterial protease (staphylokinase and streptokinase) and measuring influenza virus replication. Results indicated that interaction of extracellular proteases with the HA may activate viral infection in both experimental and in natural situations¹⁷. Even with the uncleaved Hemagglutination (HA), the influenza virus could become infectious through the host cell endoproteases, sometimes induced by enzymatic activation¹³.

In addition to the lack of understanding regarding the association mechanism of bacteria and influenza virus, some studies suggest an enhancement of respiratory infections in this situation^{3,5,16}.

Many microorganisms may represent an important source of proteases capable of a proteolytic activation of influenza virus. *Staphylococcus aureus*, *Streptococcus pneumoniae* and *S. pyogenes* are the most common bacteria in respiratory flora, that also includes *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*; the latter two species of microorganisms coming from environmental sources¹⁷. In our previous study, an increase of influenza infectivity was observed when these virus isolate samples, collected from natural hosts, were co-infected with *S. maltophilia*¹⁰.

In the present study, the co-infection influenza virus proteolytic bacteria in samples obtained from individuals presenting respiratory symptoms, including hospitalized patients presenting a situation of immune depression was studied. The ability of these bacterial species to exacerbate influenza in humans, under these conditions, has been investigated. Since there is a lack of these informations in Brazil, our objective was to verify the interaction influenza virus proteolytic bacteria in our country. However, it was not our objective, the evaluation

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of the clinical manifestation exacerbations as answers to this co-infection.

MATERIAL AND METHODS

Microorganism isolation (12): A total of 407 nasal washes were collected with sterile PBS from patients (kidney transplanted), health care workers and general population with respiratory manifestation. Subjects ranged from 12 to 30 years old, were attended at the Universidade Federal de São Paulo (UNIFESP) Hospital, between 2003 and 2005. Samples were provided to both the laboratories of Virology and Bacteriology, and the techniques were performed as follows:

1. Virological tests

1.1. Immunofluorescence technique(18): The 407 samples, as referred above, were clarified by centrifugation at 2,000g for 10 min before screening for influenza positivity by immunofluorescence technique. This was performed initially by addition of mucolytic solution to these samples, followed by centrifugation at 300-500g, and incubation for 10 min at room temperature, for fixation. After this, monoclonal antibodies to influenza A and B types and parainfluenza viruses, conjugated with fluorescein isothiocyanate, were added on a Light Diagnostic Simulflour Respiratory Screen (Biotrin®). After 15 min at 37 °C (humidity chamber) the smear samples were washed with PBS (Tween) three times, for 10 sec each, and left to dry. Material was then observed by immunofluorescence microscopy (Olympus - BX51). Among the prepared samples, a standard influenza sample was included to compare the positive antigen and fluorescent influenza antibody meshes, during the microscopic examination. All samples that presented this feature, revealing fluorescence with a brilliant - green color, are considered to be the influenza virus. The other samples, considered not to be influenza antibody reagent, present a red color, due to the Blue Evans stain used for negative test confirmation.

1.2. Chain Reaction (RT/PCR) technique:

1.2.1. RNA viral extraction (4): Viral RNA is extracted from screened influenza isolate samples technique utilizing a mixing of 250 µL of the sample and 750 µL of the Trizol reagent (Invitrogen®) and kept at room temperature for five min with addition of the 200 µL Chloroform (Merck®). The samples are mixed by vortexing for 15 sec and kept at room temperature for five min, after this they are centrifuged at 15,294g for 15 min at 4 °C. Four hundred microliters of the supernatant are removed avoiding the interfase and 500 µL of the isopropanol (Synth®), it is then mixed by vortexing for five sec. These samples are centrifuged at 15,294 g for five min at 4 °C and discarding the supernatant. To this pellet, 1,000 µL of ethanol (75%) (Synth®) are added, and mixed gently following of centrifugation at 15,294 g for 10 min at 4 °C and discarding the supernatant.

Finally, 20 µL of distilled water DNase, RNase free are added and incubated at 50 °C for 10 min. This material is stored at -70 °C until performance of the reverse transcription technique.

1.2.2. Reverse transcription/PCR (6): Five microliters of viral RNA are added to 1.0 µL of primer PcDNA (flu) and 1.0 µL of primer REV, 1.0 µL of 10 mM dNTP mix and 4.0 µL of sterile distilled water.

The reaction after heating at 65 °C for five min is quickly chilled on ice followed by adding 4.0 µL of 5X First Strand Buffer (Invitrogen®), 2.0 µL of 0.1M DTT (Invitrogen®), 1.0 µL of Rnase Out Ribonuclease Inhibitor (Invitrogen®), and incubated at 37 °C for two min. Next, it is added 1.0 µL (200 units) of RT (M-MLVRT-Invitrogen®), mixed by pipetting gently, incubated at 37 °C for 50 min and the reaction was inactivated by heating at 70 °C for 15 min. The PCR product, is the viral cDNA that should be stored at -70 °C.

PCR Reaction for Amplification: Five microliters of cDNA are amplified in a volume of 50.0 µL containing 5.0 µL PCR buffer 10X 20 mM Tris-HCl pH 8.4, 500 mM KCl, 1.5 µL 50 mM MgCl₂, 1.0 µL 10 mM dNTP, 35.1 µL H₂O DEPEC, 1.0 µL of primer PcDNA (- ; FLU), 1.0 µL of primer REV(+; Flu) and 0.4 µL (5U/µL) Taq polymerase. This reaction mixture is warmed by Thermal Cycler (Bio-Rad®) at 94 °C for 45 sec and 35 cycles of: 45 sec at 94 °C; 30 sec at 55 °C ;1.3 min at 72 °C, and 72 °C for 10 min. Finally, it is left at 4 °C for indeterminate time. The PCR amplified product is analyzed by 2.0% agarose gel (Sigma®) electrophoretic run 60V and bands stained with 0.5 µg/mL ethidium bromide, and documented by Fluorescent Table (Vilber-Lourmat®) and photographed by digital camera (Sony®). Influenza virus type A (H3N2), used as standard, will confirm the samples positivity through the bands revealed with 189 bp from the primers corresponding to no structural antigen{NS1} gene fragment of the influenza virus.

Primer PcDNA Sequence (5' to 3'): AAG GGC TTT CAC CGA AGA GG

Primer REV Sequence (5' to 3'): CCC ATT CTC ATT ACT GCT TC

1.3. Hemagglutination (HA) Test (9): Laboratory cell-adapted strain of Influenza virus A/SP/1/91 (H₃N₂) after contact, for 60 min at 25 °C, with bacterial protease samples, performed the virus hemagglutination titer, which is determined at room temperature in a microtiter system. Serial two fold dilutions of virus (25 µL) in phosphate-buffered pH 7.2 are mixed with 25 µL of a 0.5% suspension of rooster red blood cells. Hemagglutination titers are determined after one hour, unless otherwise stated, and are expressed as the reciprocal of the maximum dilution of virus that causes complete agglutination.

1.4. Cytopathic effects in cell culture (7):

1.4.1. Infection of cell culture by Influenza virus in the presence of bacterial protease: Laboratory cell-adapted strain of Influenza virus type A/SP/1/91 (H₃N₂) is used to infect monolayers of MDCK cells. In twelve wells of plastic plaque (Falcon, USA) with cell culture, it was inoculated 0.1 mL of each influenza virus dilution at 10⁻¹ to 10⁻⁴ previously contacted with bacterial protease (0.1 mL) for 60 min at 25 °C. The plaque with infected cells is left for 30 min at 33 °C, and then completed with the Leibovitz-15 medium. Controls such as influenza virus replication in absence or presence of trypsin (30 µg/mL) and normal cells are performed likewise the experimental test. Four wells are used for each test: experiment and controls. These test results hold on increase of the virus influenza infectivity titer, such as by cytopathic effect (CPE) as well by HAU, both detected after seven days of the virus inoculation, which is the end test.

2. Bacterial tests (12)

2.1. Bacterial cultivation and identification: From the total sampling of the 407 collected individuals, thirty seven samples screened by immunofluorescence as influenza positive were chosen for bacteria detection through the cultures in Tryptic Soy Broth (TSB) for 18-24 h at 35 °C - 37 °C. Samples that presented medium opacity, after this time, were submitted to the identification assay. For this, the cultured bacteria was dropped on glass smears and stained by the Gram technique and then visualized by optic microscopy. The microorganisms, of both coccoids and bacillar morphologies [being Gram (+) or (-)], were screened by biochemical methods. For Gram positive microorganism identification by catalase test, a serology test was employed using the Staphy Test commercial kit (Probac®), to identify the *Staphylococcus aureus* and negative coagulase. This positivity test is observed through the agglutination of the sheep blood sensitized by hemolysin and fibrinogen. Referring to negative test result, that lead to suggest to be *Staphylococcus* negative coagulase concerning *Streptococcus* identification, plaques (Petri) with sheep blood agar (5%) were prepared to differentiate hemolysis types. The material, previously isolated with a coccoid form, as verified by microscopy, was cultured on these plates and Optochine discs added (5 µg) (Probac®) before incubating in CO₂ atmosphere (range 5 - 10%) for 24 h at 37 °C. After this time, the bacterial culture that presented halo ≥14 mm around these discs may be considered as *S. pneumoniae*, and those which demonstrated a green color with halos are identified as *S. viridans*.

Plaques of E.M.B: Agar were used for cultivation of the Gram negative microorganism isolated and identified through biochemical assays. The *Pseudomonas* spp. can be suspected by betalactams antibiotic resistance.

2.2. Protease Test-Agar Casein Assay (3): Bacterial isolates cultured in Tryptic Soy Broth (TSB) were centrifuged at 12,000g for 15 min at 4 °C and filtered through a Millipore 0.45 µm pore-diameter syringe filter. Clarified supernatant was tested for proteolytic activity on casein agar plates. Casein agar plates consisted of 25 mM Tris (pH 7.2), 150 mM NaCl, 0.6% casein (Sigma technical grade) and 1% Bacto Agar (Difco®). Aliquots (10 µL) of culture supernatants are placed in 3 mm diameter wells cut in the casein agar and incubated at 37 °C for 18h. The plates are overlaid with 3% acetic acid, and proteolytic activities are noted as a zone of clearing around the sample well. Trypsin (1 µg/mL) was used as a positive control standard. Caseinolytic activity of the proteolytic bacteria, also relative to trypsin used as a control, was determined by measuring the diameter of the proteolytic zones around each well.

RESULTS

Through the virological tests, among of the 407 samples collected from individuals presenting respiratory manifestations, thirty seven (9.09%) samples were chosen that had been previously demonstrated as influenza virus positive by immunofluorescence, in whose samples the parainfluenza was not detected. These samples were identified as being 25 (67.56%) samples of type A and 12 (32.43%) of type B. The fluorescent positive influenza sample is shown in Fig. 2. The RT/PCR technique characterized the influenza virus in some samples previously screened for this virus. The electrophoretic bands revealed that the isolated

influenza virus strains were compatible with standard influenza A/SP/1/91 (H₃N₂). A PcDNA(flu) primer was used to amplify a 189 bp fragment of the non-structural (NS-1) gene of influenza virus in Fig. 3.

Of the eighteen samples (48.64%) presenting *Staphylococcus* negative coagulase, *Staphylococcus aureus*, *Streptococcus viridans*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* that were obtained from thirteen (35.13%) influenza types A and five (13.31%) B samples and characterized by the bacteriological tests recommended for this type of investigation (Table 1 and Fig. 1). The Agar Casein test showed three samples of these bacteria isolates with protease action, as verified by the zone of clearing around each bacteria sample well. Trypsin represents the proteolytic action control. The *Staphylococcus* negative coagulase (isolated) and *S. pneumoniae* negative coagulase from ATCC represent the lack of the proteolytic action (Fig. 4).

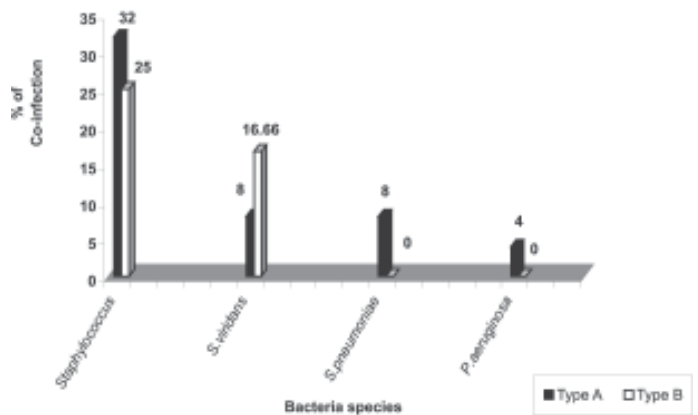


Fig. 1 - Percentage of *Staphylococcus* spp., *Streptococcus viridans*, *S. pneumoniae* and *Pseudomonas* spp. co-infecting with influenza A and B viruses in nasal washes from patients exhibiting manifestations of respiratory ailment.

Using laboratory cell-adapted strains of influenza virus A/SP/1/91 (H₃N₂), contacted or not previously with bacteria proteases from *Pseudomonas aeruginosa* (sample 68); *Staphylococcus aureus* (sample 158); *Streptococcus viridans* (sample 342); *Streptococcus pneumoniae* (samples 344 and 356), the increase in infection by this virus, as in MDCK cells, was observed by their cytopathic effect (CPE) as well as by their hemagglutinin titers. Trypsin was used to demonstrate enzymatic action on the influenza hemagglutinin cleavage, with viral exacerbation. These results are demonstrated in Table 2 and Fig. 5.

Bacteria such as *Pseudomonas aeruginosa* (sample 68) co-infecting influenza samples, as well as in combination with cell-adapted influenza A (H₃N₂) are represented in Fig. 6a and 6b.

DISCUSSION AND CONCLUSION

Bacteriological and Virological tests demonstrated that the samples collected by nasal wash from individuals presenting respiratory manifestations, revealed bacteria and influenza virus presence concomitantly. The influenza type A was more incident than type B, as well the *Staphylococcus* reached the higher percentage, in these evaluated samples.

Table 1
Relation of the detection of influenza virus and bacteria in nasal washes collected from individuals presenting respiratory manifestations

| Screened samples | Tests | | Bacteriological identification Microorganism |
|------------------|----------------------------------|-------------|--|
| | Imunofluorescence Influenza type | | |
| | A | B | |
| 1 | + | - | N |
| 2 | + | - | <i>Staphylococcus</i> negative coagulase |
| 5 | - | + | <i>Streptococcus viridans</i> |
| 7 | - | + | <i>Streptococcus viridans</i> |
| 16 | + | - | N |
| 24 | + | - | N |
| 25 | - | + | N |
| 27 | - | + | N |
| 28 | + | - | N |
| 29 | - | + | <i>Staphylococcus aureus</i> |
| 30 | + | - | <i>Staphylococcus aureus</i> |
| 36 | - | + | N |
| 38 | + | - | N |
| 39 | - | + | N |
| 40 | + | - | N |
| 68 | + | - | <i>Pseudomonas aeruginosa</i> |
| 116 | - | + | <i>Staphylococcus</i> negative coagulase |
| 130 | - | + | N |
| 142 | + | - | <i>Staphylococcus</i> negative coagulase |
| 147 | + | - | <i>Staphylococcus</i> negative coagulase |
| 149 | - | + | N |
| 151 | - | + | N |
| 152 | + | - | <i>Staphylococcus aureus</i> |
| 155 | - | + | <i>Staphylococcus</i> negative coagulase |
| 156 | + | - | N |
| 158 | + | - | <i>Staphylococcus aureus</i> |
| 159 | + | - | N |
| 327 | + | - | <i>Staphylococcus aureus</i> |
| 340 | + | - | N |
| 342 | + | - | <i>Streptococcus viridans</i> |
| 344 | + | - | <i>Streptococcus pneumoniae</i> |
| 346 | + | - | N |
| 356 | + | - | <i>Streptococcus pneumoniae</i> |
| 358 | + | - | <i>Staphylococcus aureus</i> |
| 366 | + | - | N |
| 367 | + | - | <i>Streptococcus viridans</i> |
| 375 | + | - | N |
| 37 | 25 (67.56%) | 12 (32.43%) | 18 (48.64%) |

N - Negative.

Proteolytic bacteria, such as *Pseudomonas* spp., *Staphylococcus* spp. and *Streptococcus* spp., were identified in these samples. The protease action of these bacteria was demonstrated by the assay performed with cell-adapted influenza virus A (H₃N₂) that was exacerbated through contact with the bacteria isolates. Influenza

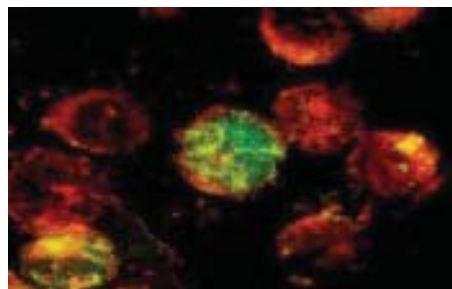


Fig. 2 - Immunofluorescence micrograph of the influenza virus in nasal washes from patients exhibiting symptoms of respiratory ailment. - Green = Positive Antibody reaction to the Influenza virus; - Red = Negative Antibody reaction to the Influenza virus.

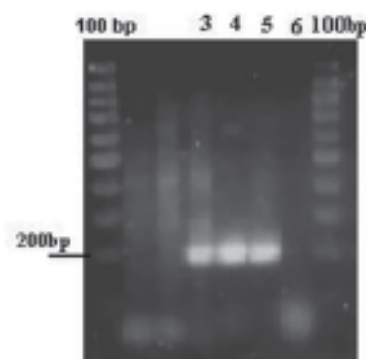


Fig. 3 - Characterization by RT/PCR of the influenza virus in nasal washes from patients presenting respiratory ailments manifestations. Lane 3 - Sample # 68; Lane 4 - Sample # 342; Lane 5 - Influenza virus type A(H3N2); Lane 6 - Distilled water.

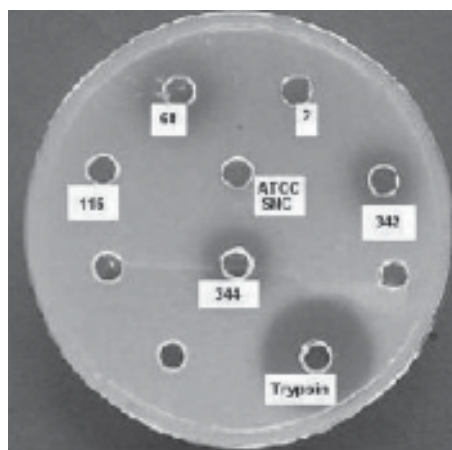


Fig. 4 - Agar-Casein Test - Protease-positive Bacteria co-infecting samples of influenza virus isolate. #68 - *Pseudomonas aeruginosa* - Protease Positive; #342 - *Streptococcus viridans* - Protease Positive; #344 - *Streptococcus pneumoniae* - Protease Positive; #2 - *Staphylococcus* negative coagulase; #116 - *Staphylococcus* negative coagulase; ATCC/SNC - Protease Negative Control; Trypsin - Protease Positive Control.

Table 2

Cell-adapted influenza type A (H₃N₂) strain infectivity (TCID₅₀) and hemagglutinating titer exacerbations by co-infection with proteolytic bacteria

| Dilution Influenza + Bacteria Protease | Influenza Infectivity by CPE Sample | | | | Virus Control Trypsin |
|---|--|-------------------|-------------------|-------------------|-----------------------------|
| | #68 | #342 | #344 | #356 | |
| 10 ⁻¹ | 70% | 100% | 100% | 100% | 75% |
| 10 ⁻² | 65% | 70% | 70% | 70% | 60% |
| 10 ⁻³ | 60% | 60% | 65% | 65% | 40% |
| 10 ⁻⁴ | 40% | 50% | 50% | 60% | 30% |
| 10 ⁻⁵ | 30% | 40% | 40% | 40% | 10% |
| TCID ₅₀ | 10 ^{3.4} | 10 ^{3.7} | 10 ^{4.0} | 10 ^{4.0} | 10 ^{2.8} |
| TITER (HAU) | 25 | 28.8 | 36 | 38 | 8 |

CPE - Cytopathic Effect; TCID₅₀ - Tissue Cell Infectious Doses; HA - Hemagglutination; HAU - Hemagglutinating Unit.

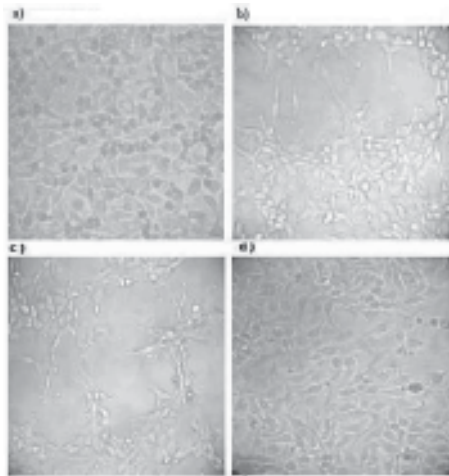


Fig. 5 - Cytopathic effect of the cell-adapted influenza A virus (H3N2) with and without protease in MDCK cells. a) - Normal MDCK cells; b) - Influenza X Bacterial Protease; c) - Influenza X Trypsin; d) Influenza without Protease.

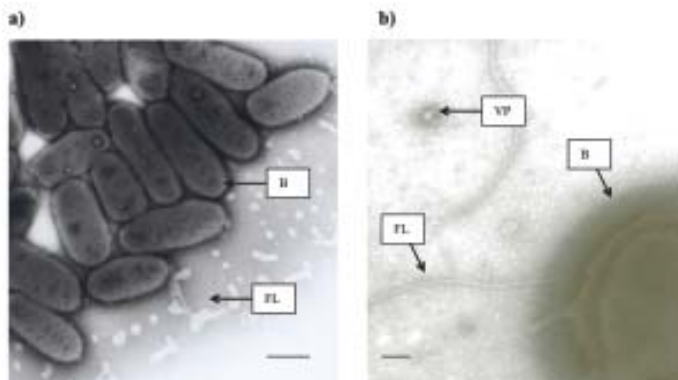


Fig. 6 - Electron micrograph of proteolytic bacteria present in influenza samples isolated from hospitalized patients. a) Sample #68 - *Pseudomonas aeruginosa*; B = Bacteria; FL = Flagellum; Magnification = 15,455 X; Scale bar = 100 nm. b) Cell-adapted influenza virus A (H3N2) in contact with bacteria; B = Bacterium; VP = Viral Particle; FL = Flagellum; Magnification = 35,089 X; Scale bar = 100 nm.

infectious and hemagglutinin titers were amplified by co-infecting with proteolytic bacteria, compared with trypsin presence or absent in these tests. Then, considering this finding with regards to exacerbation of the influenza virus infectivity by bacterial protease, observed *in vitro*, lead to suggest the damage that this co-infection causes to the hosts.

Thus, observations since the early eighties by authors such as TASHIRO *et al.*, 1987, BULGAKOVA *et al.*, 1987, demonstrate these bacteria as being responsible for fatal disease in mice lungs, when co-infected with influenza virus. These studies were continued over twenty decades¹¹, demonstrating the lethal synergism between influenza virus and *Streptococcus pneumoniae* that led to 100% of mortality in mice used as an experimental model. This finding has been related not only in animal models; a synergism between bacteria-influenza virus has been reported, and also observed in children with pneumococcal pneumonia that preceded influenza infection¹⁴ at the Centers of Disease Control, in Atlanta, USA. Once again, hospitalized children with Community Acquired Pneumoniae (CAP), provoked by influenza infection concomitant with *Streptococcus pneumoniae*, were evaluated by PONS-CATALANO *et al.*, 2003. Recently, BEADLING & SLIFKA, 2004, reported that bacterial sepsis was a leading cause of death in the United States, where the mechanisms of lethal synergism between bacteria and influenza virus are responsible for the sepsis in these hospitalized individuals. Authors suggest that preventive measures, including vaccination against influenza and aggressive anti-microbial therapy, early on the course of infection, may reduce the morbidity and mortality by sepsis.

Based on our data and the other reports cited, it may be concluded that co-infection between these microorganisms exacerbates respiratory infections. This situation may be aggravated in hospitalized individuals with immune depression, such as in some patients evaluated in this work. The clinical exacerbations had been not investigated in this work. Even so, this could occur among the evaluated subjects. Therefore, beyond the cited measurements suggested for this preventive control, it is important to currently investigate the most frequent bacterial species that could be associated with influenza virus infection, in the hospital environment, to offer the appropriate medical action against these infectious agents.

About this issue, natural antioxidants from spice extracts are being used as anti-microbials in these bacterial isolates, following our previous findings on the anti-viral action of phenolic compounds, present in natural antioxidants, including the rosemary spice⁸. In addition, inhibitors against bacterial proteinase are being recommended to ameliorate the anti-pathological processes, with reduction of growth rate of the invading organism, allowing the host defense response to be re-established²⁰. These findings require further studies, to investigate the synergism between cell endoproteases and microbial proteases in the cleavage of influenza hemagglutinins, as well the cell-adhesion bacteria, favored by the influenza virus, which certainly could potentiate the clinical manifestations of respiratory infections.

The first step of this study had been lead to investigate the incidence of co-infection between virus and proteolytic bacteria, with stand out the influenza infection exacerbation, and the next step will be expended with regards to the bacterial infection exacerbation, under viral co-infection conditions.

RESUMO

Vírus influenza e bactéria proteolítica co-infectantes em trato respiratório de indivíduos com manifestações respiratórias

O papel da bactéria proteolítica na exacerbação do vírus influenza tem sido demonstrado em hospedeiros naturais como porcos e humanos. Foram coletadas 407 amostras do trato respiratório de indivíduos apresentando manifestações clínicas, durante a estação da influenza (2003-2005) na cidade de São Paulo. Este trabalho teve como objetivo avaliar a incidência de determinadas bactérias que junto com vírus co-infectarem o trato respiratório humano. Testes bacteriológicos, e virológicos com imunofluorescência (IF), RT/PCR e hemaglutinação (HA) foram usados nas investigações viral e bacteriana. Pelo teste de IF foram selecionadas trinta e sete (9,09%) amostras positivas para o vírus influenza. A presença do vírus influenza foi confirmada pela técnica de RT/PCR. Pelos testes bacteriológicos e do agar caseína, verificou-se que 18 (48,64%) dos indivíduos foram infectados com bactérias proteolíticas tais como *Staphylococcus* spp., *Streptococcus* spp. e *Pseudomonas* spp. Destas amostras, 13 (35,13%) foram co-infectadas com vírus influenza tipo A, e 5 (13,51%) com influenza tipo B. No experimento *in vitro* com influenza e *S. aureus*, detectou-se aumento do título hemaglutinante deste vírus, após contacto de 30 min a 25 °C. Os resultados obtidos revelaram a ocorrência de co-infecção com bactéria proteolítica e vírus influenza nos indivíduos avaliados. Estes achados corroboram com a investigação do sinergismo, entre bactéria e vírus, que poderia ser capaz de potencializar infecção respiratória, aumentando os riscos aos hospedeiros.

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