Nasopharyngeal Lactate Dehydrogenase Level as a Predictor of Bronchiolitis Severity

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Abstract: Bronchiolitis is inflammation of smallest air passages of the lungs. Respiratory syncytial virus is the most common cause. Lactate dehydrogenase (LDH) is a membrane associated protein, when found extracellularly indicated cell damage and inflammation. We measured LDH in nasopharyngeal aspirate from a cohort of children with bronchiolitis in relation to disease severity. Patients and methods: It is a prospective study enrolled 73 patients with age less than 24 months presenting to emergency department with bronchiolitis, excluding those with previous wheezing or eczema, gastroesophageal reflux disease, congenital heart diseases, family history of asthma. Nasopharyngeal aspirate was done for all patients. Parameters of disease severity included the need for hospitalization, oxygen supplementation, intravenous fluids, and requirement for intubation. Viral identification was done by reverse-transcriptase polymerase chain reaction. Samples were tested also for cytokines and chemokines, total protein by the bicinchoninic acid method and lactate dehydrogenase. Apoptosis Quantification was measured with Caspase-Glo-3/7 kit. Results: 73 patients enrolled with median age 6.5 months. RSV infection was the cause in 66%. No differences in nasopharyngeal aspirate LDH concentrations according to age, gender, environmental exposure to tobacco smoke, day care attendance, or exposure to young children. T helper1 and Th2 cytokines detected in nasopharyngeal aspirate fluid samples were strongly correlated with LDH in nasopharyngeal aspirate fluid samples. Patients receiving oxygen for > 24 hours had lower nasopharyngeal aspirate LDH levels, and high concentrations of nasopharyngeal aspirate LDH (≥365 U/ml) were associated with 79% reduction in the need for admission.

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1. Introduction

Bronchiolitis is inflammation of the bronchioles, the smallest air passages of the lungs. It usually occurs in children less than two years of age with the majority being aged between three and six months, annual bronchiolitis hospitalization among infants younger than 1 year has been assessed at 31.2/1000 [1]. It is predominately a viral disease and the number of cases where no agent is identified has decreased over the years with the widespread use of more sensitive diagnostic techniques [2] and the recognition of new viruses [3-7]. Respiratory syncytial virus is the chief cause of hospitalization for respiratory tract illness in young children [8-9] and the most common agent recovered from children with bronchiolitis [10]. During the epidemic season, up to 90% of this acute wheezing disease has been attributed to respiratory syncytial virus [10].

Despite identified risk factors for severe disease, the difficulty and uncertainty of determining the appropriate level of supportive care for children with bronchiolitis is well documented by the large variability in hospital admission practices. There is a need to have a biochemical marker of disease severity to assist the clinician in determining hospitalization requirement in children with bronchiolitis. Lactate dehydrogenase (LDH) may be such a biochemical marker because of its relation to tissue injury. LDH is a membrane associated protein involved in the oxidation of various organic substrates [11]. It is present in all major organ systems and when found extracellularly indicated cell damage and inflammation [12]. Measurement of LDH in bronchoalveolar fluid is commonly done as a surrogate for the presence of leukocytes and overall inflammation. In children with RSV bronchiolitis, neutrophils account for over 90% of total cells in nasopharyngeal aspirate and more than 70% in bronchoalveolar fluid [13-14]. LDH activity in nasopharyngeal aspirate has not been reported for children with bronchiolitis.

We postulated that LDH would directly correlate with the proinflammatory immune response observed in bronchiolitis, and that it would be a useful biochemical marker of disease severity. We measured LDH in nasopharyngeal aspirate specimens

from a cohort of children with bronchiolitis in relation to disease severity.

2. Material and Methods

Study design: Study consisted of a prospective investigation of children < 24 months old presenting to the emergency department with bronchiolitis from September 1, 2008 to February 28, 2010. Infants were excluded if having any of the following criteria: previous wheezing, history of eczema, regular use of bronchodilator or anti-inflammatory medication, gastroesophageal reflux disease, congenital heart diseases, congenital anomalies of the chest or lung, chronic lung disease of prematurity, family history of cystic fibrosis or family history of asthma, as all these condition can alter inflammatory response and hence inflammatory mediators. Sample size was 100 patients but 18 patients excluded due to incomplete chart and 9 for improper sample collection or missed sample. The study was approved by the local review board and ethical committee.

Bronchiolitis was defined as an acute lower airway infection (presence of tachypnea, crackles, wheezing, and/or retractions) and a history of a previous upper-airway infection (defined as nasal congestion and/or rhinorrhea) diagnosed by experienced pediatric ED physicians. The decision to hospitalize a patient was made by the attending physicians following standard clinical pathways. Parameters of disease severity included the need for hospitalization, oxygen supplementation, intravenous fluids, and requirement for intubation.

Sample collection: On arrival to the ED, consent was obtained from parents or caregiver. Physician conducted a physical examination, recorded patient demographic -including age, sex, weight, history of smoking exposure, family history of atopy-and clinical information. A nasopharyngeal aspirate sample was obtained by using a standard procedure. Viral transport medium was added to a final 1:1 dilution. Aliquots were made, snapped-frozen in a dry ice/alcohol bath, and stored at -70 ℃.

Detection of viruses: Viral identification was done by reverse-transcriptase polymerase chain reaction (RT-PCR). All specimens examined using specific primers and fluorescent probes for respiratory viruses including RSV, human rhinovirus, human metapneumovirus (HMPV).

RSV Quantification: RSV concentration was quantified by using 2 methods. First, all RSV-positive samples were titrated by plaque assay in duplicates and reported as plaque-forming units per milliliter. Second, all samples were analyzed by real-time RT-PCR blinded to quantitative culture results. Calibrated standards for each target were assayed in parallel to the samples. The number of cycles required for the fluorescent signal to reach a threshold level was recorded as the crossing threshold. Therefore, the lower the crossing threshold, the higher the amount of genomic material and vice versa.

Cytokine/Chemokine determination: Nasopharyngeal aspirate samples were tested for cytokines and chemokines with the Bioplex Human Cytokine 17-plex panel (Bio-Rad Laboratories [Herules, CA]) following manufacturer instructions. The lower limit of detection for all cytokines and chemokines was 1pg/ml.

Total Protein Measurement: Total protein was quantified in nasal secretions by the bicinchoninic acid method (Pierce BCA Protein Assay Kit, Thermo Scientific, Rochford, IL) following the manufacturer instructions and expressed as micrograms per milliliter.

Lactate Dehydrogenase Determination: Total LDH activity was tested in nasopharyngeal aspirate samples. Samples were diluted 1:1 in phosphate-buffered saline, and assayed in duplicates following protocol instructions (Cytotoxic Detection Kit, Roche Applied Science [Indianapolis, IN]). Absolute values were calculated.

Apoptosis Quantification: LDH release may be a marker of cell destruction caused by virus or the host cellular immune response, or may be because of apoptosis induced by virus or the innate immune response. To measure apoptosis in nasal secretions, the Caspase-Glo-3/7 kit (Promega [Madison, WI]) was used. The luminescence was measured with a FLUOstar OPTIMA microplate reader (BMG Labtech [Cary, NC]) and expressed in relative luminescence units.

Statistical Analysis: LDH concentrations (in nasopharyngeal samples) and caspase activity were normalized by log10 transformation. The statistical evaluation of normally distributed continuous variables was performed with Student's t test or 1-way analysis of variance as appropriate. Differences of proportions of non-parametric data were compared with the X2 test. Correlations were calculated by Pearson's or Spearman's coefficients as appropriate. Statistical significance was established with 2-tailed tests and αlevel of $P < .05$.

The association between hospitalization (defined as a hospital stay of >24 hours) and independent variables was investigated with multivariate logistic regression analyses. Factors identified by univariate analysis were included in the model if the association with the outcome had a P value of <.25. The model contained the following categorical variables: age (≤3 or >3 months); hypoxia (yes or no); and administration of intravenous fluid (yes or no). The nasopharyngeal LDH concentration

and the caspase 3/7 activity were classified in quartiles (≥75% or <75%).
All statistical analyses were performed by using Stata 10.0 for Windows (Stata Corp, College Station, TX).

3. Results

Etiologic Agents of Bronchiolitis in the Population: In this prospective study, 73 pediatric patients with bronchiolitis were enrolled from emergency department of King Fahd Hospital. Median age at presentation was 6.5 months (range: 0.34-22.9), and 58.9%were boys (43 patients). Median duration of illness at the time of presentation to the ED was 2.5 days (mean ± SD: 5.2 ±4.2 days).
RSV infection was the leading cause of bronchiolitis, present in 66% (48 patients) of individuals. Rhinovirus was second in frequency, being detected in 19% of cases (14 patients). And 6 patients (8.2%) had dual infections.

Table 1: Viral Etiology of Bronchiolitis in the Study Population

Virus	Number of patients
RSV	48
Rhinovirus	14
RSV+ rhinovirus	6
Adenovirus	1
Parainfluenza	1
Picornavirus	1
Cytomegalovirus	1
No virus	1

The proportion of RSV infected subjects was comparable between those hospitalized versus those discharged from the hospital from the ED (P=.3).

Demographic and Clinical Factors: There were no differences in nasopharyngeal aspirate LDH concentrations according to age, gender, environmental exposure to tobacco smoke, day care attendance, or exposure to young children. The detection of a virus (P=.01 versus no virus) or the presence of dual viral infection (P=.02 versus single or no virus infection) were associated with higher concentration of nasopharyngeal aspirate LDH. Children with RSV infection had greater nasopharyngeal aspirate LDH compared with children not infected with RSV (P< 0.001). The difference persisted after excluding 6 patients with dual infection with RSV and another viral pathogen (mean log10 NP LDH: 2.5 [95% confidence interval (Cl): 1.98-2.32] vs 1.58 [95% Cl: 1.24-1.93], for patients with single RSV infection versus patients without RSV detection, respectively; P=.001).

Table 2: Demographic and Clinical Features of Study Population and LDH Concentration in Nasopharyngeal Aspirate

Condition	n	Mean NP LDH Concentration, Log10 (95% CI), U/mL	P
Age (month)			.37
0-3	21	2.01 (1.71–2.04)	
3-6	10	2.13 (1.81–2.49)	
6-12	23	2.01 (1.71–2.19)	
12-24	19	1.743 (1.12–2.36)	
Gender			.85
Male	44	1.99 (1.80–2.10)	
Female	29	2.00 (1.69–2.06)	
Environment			
Exposure to cigarette smoke	45	1.93(1.59–2.29)	
No Exposure to cigarette smoke	28	2.02 (1.85–2.19)	.63
Daycare	43	1.98 (1.59–2.36)	
No Daycare	30	2.04 (1.84–2.21)	.84
Exposure to children <5 y old	40	2.04 (1.85–2.23)	
No Exposure to children <5 y old	33	1.95 (1.65–2.25)	.61
Duration of illness (day)			
1-3	22	2.05 (1.65–2.39)	
3-6	40	2.00 (1.75–2.25)	.28
6-10	11	2.20 (1.94–2.50)	
Virus detection			
No virus identified	1	1.41 (0.88–1.94)	
Single infection	66	2.06 (1.89–2.21)	.001
Dual infection	6	2.40 (2.14–2.65)	
RSV status			
RSV	48	2.20 (2.05–2.35)	
Non-RSV	24	1.61 (1.28–1.95)	<.001

Nasopharyngeal LDH, Total Protein, and RSV concentration: Nasopharyngeal aspirate LDH concentration was determined in the study population. Levels were not significantly different between children in the following age group: <3 to 5, 6 to 11, and 12 to 24 months. No correlation was observed between concentration of LDH in serum and

nasopharyngeal aspirate fluid (Spearman's p =0.06; P=.6).

We have observed in vitro that increased RSV replication in respiratory epithelial cells results in greater concentrations of LDH in the supernatant, and that this effect is potentiated by the presence of proinflammatory cytokines. The correlation between nasopharyngeal LDH and infectious RSV measured by plaque assay failed to reach statistical significance (P=.05); however, a significant correlation was noticed when the RSV load was quantified by subgroup-specific real-time PCR (r=0.58, P<.001).

Total protein content in the nasopharyngeal aspirate fluid may be a combination of proteins released during virus-induced epithelial cell injury and the host immune response. As expected, a significant correlation was observed between nasopharyngeal aspirate total protein and nasopharyngeal LDH concentration (r=0.48, P<.001)

Cytokines, Chemokines and Apoptosis: T helper, 1 cytokines (interkeukin 2 (IL-2), interferon γ, tumor necrosis factor α, and IL-1β). Th2 cytokines (IL-4, IL-6, IL-10) and other proinflammatory cytokines (macrophages, inflammatory protein 1β, monocyte, chemoattractant protein 1, IL-8, granulocyte-macrophage-colony-stimulating factor [GM-CSF], and granulocyte-colony-stimulating factor) detected in nasopharyngeal aspirate fluid samples were all strongly correlated with LDH in nasopharyngeal aspirate fluid samples. A positive linear correlation is observed between nasopharyngeal aspirate LDH and nasopharyngeal aspirate caspase 3/7, a measure of apoptosis activity (r=0.75; P<.001).

Analysis of Indicators of Severe Bronchiolitis: Classic indicators of bronchiolitis severity are shown in table 2, with their respective levels of nasopharyngeal aspirate LDH. Children with bronchiolitis who were discharged from the hospital from the ED had significantly higher nasopharyngeal aspirate LDH concentrations than those who were admitted for supportive care (2.41 vs 1.79 U/ml; P=.002) Hospitalization occurred in 82%, 71%, and 50% of children aged <3 to 5, 6 to 11, and 12 to 18 months respectively.

Approximately half of the patients who were hospitalized required supplemental oxygen. Patients receiving for > 24 hours had lower nasopharyngeal aspirate LDH levels compared with children who received ≤ 24 hours of oxygen, although the difference disappeared when considering only those children who were hospitalized. Patients with high level of nasopharyngeal aspirate LDH received shorter duration of oxygen supplementation or none (Wilcoxon rank-sum test: z=2.76; P=.006). Intubation was not associated with differences in nasopharyngeal aspirate LDH, only 5 patients required mechanical ventilation. Collectively, these data indicate that a more severe clinical presentation leading to hospitalization was associated with lower levels of nasopharyngeal aspirate LDH concentrations.

Table 3: Univariate Analysis for Indicators of Severe Bronchiolitis

Severity Parameter	n	Mean NP LDH concentration (95% Cl)	P
Hospitalization			.002
Yes	50	1.84 (1.64-2.03)	
No	23	2.35 (2.12-2.57)	
Oxygen supplementation			.03
≤24h	50	2.03 (1.90-2.5)	
>24h	23	1.79 (1.50-2.01)	
Mechanical ventilation			.40
Yes	5	1.75 (0.86-2.65)	
No	68	2.01 (1.85-2.17)	
Intravenous fluid			.12
Yes	30	1.85 (1.63-2.10)	
No	43	2.10 (1.81-2.30)	
Intravenous fluid			.06
≤24h*	53	2.07 (1.90-2.25)	
>24h	20	1.73 (1.44-2.03)	
Feeding intolerance**			.09
Yes	26	1.09 (0.96-1.99)	
No	47	1.98 (1.8-2.01)	

*Include patients who did not receive intravenous fluid

**Feeding intolerance is considered when child is unable to take orally for 24 hours or more or taking orally less than 50% of his usual or with vomiting following 1/2 number of feeds.

Odds ratios for bronchiolitis severity leading to hospitalization according to multivariate analysis: Quartile distribution of nasopharyngeal aspirate LDH was as follows low-quartile group, <3.10 U/ml; midquartile group, 3.10 to 365; high-quartile group, ≥365. The univariate analysis identified the following variables for inclusion in the multivariate logistic regression analysis for hospitalization: presence of fever (odds ration [OR]: 0.47; P=.12), need for intravenous fluid (OR: 12.9; P<.001), detection of viral pathogen (OR: 0.39; P=.18), age ≤ 3 months (OR: 3.2; P=.02), Nasopharyngeal aspirate LDH concentration above 75% percentile (OR: 0.46; P=.12), and presence of hypoxia (OR: 9.7; P=.03). The final multivariate

prediction model for hospitalization among children presenting to the ED with bronchiolitis is presented in table 4.In this model, age ≤ 3 months and the presence of hypoxia were associated with ~5- and 10-fold increase in hospitalization, respectively. High concentrations of nasopharyngeal aspirate LDH (≥365 U/ml) were associated with 79% reduction in the need for admission (P=.011).

Table 4: Correlation between Nasopharyngeal Aspirate LDH and Inflammatory Markers

Inflammatory	Correlation Coefficient	P
Th 1 cytokinnes		
• IL-2	0.43	<.001
• Tumor necrosis factorα	0.78	<.001
• Interferon ɤ	0.79	<.001
• IL-1β	0.83	<.001
Th2 cytokines		
• IL-10	0.60	<.001
• IL-4	0.68	<.001
• IL-6	0.74	<.001
Chemokines and other proinflammatory cytokines		
• GM-CSF	0.64	<.001
• Monocyte chemoattractant protein 1 (CCL4)	0.67	<.001
• Granulocyte colony–stimulating factor	0.77	<.001
• Macrophage inflammatory protein 1 (CCL2)	0.79	<.001
• IL-8 (CXCL8)	0.85	<.001

The final model gave an area under the receiver operating characteristic (ROC) curve of 0.87, with a Pearson goodness-of-fit test statistic of 12.95 (P= .12). Assuming a predicted probability of hospitalization cutoff value of 0.5, the model correctly classified 78.9% of patients when all observations were available for inclusion in the logistic regression. Twelve patients would have been incorrectly sent home (false-negative rate 16%).

Table 5: Multivariate logistic model to ascertain factors related to hospitalization among children treated in the ED for bronchiolitis.

Parameter	OR	(95% Cl)	P
Age ≤ 3m	5.44	1.41–20.90	.012
Hypoxia	10.71	2.54–44.88	.001
Need for intravenous fluid	12.15	1.08–135.61	.041
Log10 NP LDH ≥ 2.56U/ml	0.18	0.04–0.67	.010

The sensitivity, specificity, positive predictive value, and negative predictive value were 80%, 76%, 89%, 65%, respectively.

4. Discussions

The description of LDH in nasopharyngeal aspirate of young children with bronchiolitis in literature is infrequent. Nasopharyngeal LDH was found to be a significant independent biochemical marker in reducing the risk for hospitalization. We observed that nasopharyngeal LDH levels were significantly higher in children sent home from the emergency department compared with those admitted for bronchiolitis (P=.002) and strongly correlated with the apoptosis marker caspase 3/7 and with cytokines and chemokines involved in the innate and adaptive immune response. The detection of viruses was associated with higher nasopharyngeal LDH values. It is important of note that nasopharyngeal LDH did not correlate with serum LDH, implying that nasopharyngeal LDH originated from cellular events that occurred in the respiratory airways.

We found no correlation between nasopharyngeal and serum LDH. This indicated that LDH in nasal secretions is unlikely to be derived from capillary extravasation of protein into the airways. Authors evaluating LDH concentration in bronchoalveolar fluid concluding that its source is different from serum supported this also [22-23].

When considering the relation of nasopharyngeal to serum LDH as a ratio, a factor ≥0.6 was more likely to be present in children being sent home from emergency department compared with those admitted for supportive care (P=.003). Thus, it is reasonable to hypothesize that LDH originates from widespread airway epithelial cell injury and apoptosis or from polymorphnuclear present in the nasopharyngeal aspirate fluid. The strong correlation between nasopharyngeal LDH, IL-8 and GM-CSF supports polymorphnuclear cell as source for LDH, whereas the near linear correlation with caspase 3/7 suggests apoptosis is contributing to nasopharyngeal LDH. A better understanding of the

source of nasopharyngeal LDH may provide additional information on the early innate mechanisms involved in virus-induced bronchiolitis.

RSV is the major etiologic agent of bronchiolitis and our data reflect this fact with 66% of cases attributed to RSV. Intuitively, elevated concentration of RSV should be closely linked to more severe bronchiolitis disease. Previous studies support this theory; El Saleeby et al [27] analyzed 218 infants infected with RSV who were hospitalized during 5 winter seasons. Viral loads were more significantly associated with requirement for intensive care and respiratory failure and faster RSV clearance was independently associated with shorter hospitalization.

Wright et al. [29] examined RSV titers in nasopharyngeal samples from 77 infants who were hospitalized with bronchiolitis, and did not find demonstrable correlation between viral titer and severity of illness. Buckingham et al. [26] studied infants with RSV infection who were hospitalized. Mean nasopharyngeal concentration were significantly higher in patients who required mechanical ventilation (5.06 ± 0.34) compared with those who did not (3.91 ± 0.35).

Martin et al. described patients with respiratory infection attributed to RSV where, after adjusting for age and the presence of comorbidities, the frequency of hospitalization decreased with each log increase in viral load (OR: 0.8 [95% Cl: 0.7-0.997]; P=0.47).The strong inverse correlation we observed between nasopharyngeal aspirate LDH and RSV viral load (as quantified by real-time PCR) was not present when only infectious virus was assessed with the plaque assay. We attribute this discrepancy to the possible presence of RSV neutralizing antibodies in nasal secretions (disproved by Wright et al[29])or an increased precision of [32] PCR over traditional methods (disproved by Bae et al[31]).

Significant correlations were observed between nasopharyngeal aspirate LDH and various cytokines and chemokines, which may reflect the activation of parallel biological responses related to viral infection and innate and adaptive immune system activation. Aberrant Th2 immune responses and mutations in genes involved in the innate immune system have been linked to RSV bronchiolitis susceptibility and severity. However, recent data suggest that cytokine levels do not necessarily correlate with disease presentation [32] showed that infants with fatal RSV lower respiratory tract disease succumbed to overwhelming viral infection and epithelial cell apoptosis and not to a cytokine storm. Lung sections were characterized by a paucity of lymphocytes and natural killer cells, unchecked viral replication, and apoptostic crisis. These recent studies support the concept of a protective effect derived from a robust innate immune response during and episode of RSV bronchiolitis, where inflammatory markers inversely correlate with disease severity.

To date there is no biochemical marker predictive of disease severity in children with bronchiolitis. In our study, the sensitivity and specificity of nasopharyngeal LDH for predicting hospitalization was 80% and 75% respectively.
These values are comparable to many of the-point-care tests using in diagnosing a viral infection. The LDH assay is easy to perform, inexpensive, and available in most clinical laboratories. At our institution, the expected turn-around time for serum LDH is 1 hour, and nasopharyngeal aspirate samples should not be treated differently. Currently the presence of hypoxemia, significant respiratory distress, and the clinical judgment are the main consideration for determining the need to hospitalize a child with bronchiolitis. Having a validated biochemical marker predictive for hospitalization can provide another objective parameter to the physician, and would be valuable in difficult-to-assess cases.

A limitation of our study was that data were collected during seasons when RSV was most prevalent (and, therefore, having more weighted impact on the results) and originated from a single center. Also, a single nasopharyngeal sample was obtained from each patient, and severity of disease was determined by the final disposition of the patient and the need for therapeutic interventions. Therefore, disease progression may have occurred in patients who were discharged from the hospital.

We recognize that repeat sampling and/or longer follow-up of these patients would strengthen the conclusions. Despite the limitations, our results were consistent for multiple clinical outcomes and support the validity of our conclusions.

This study was strengthened by our comprehensive effort to characterize viral agents and the assessment of the complex interplay between RSV concentrations, the inflammatory response, patient age and severity of bronchiolitis. In addition we concluded children with mild to severe bronchiolitis in the ED setting, which was critical for a more comprehensive analysis. Additional evaluation of our hypothesis is warranted and will add to a better understanding of the immunopathogenesis of bronchiolitis.

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