Original Research

Evaluation of oxidative stress biomarkers for differentiating bacterial and viral infections: a comparative study of glutathione disulfide (GSSG) and reduced glutathione (GSH)

Zaki Milhelm[1,2], Oana Zanoaga[2], Laura Pop[2], Andrada Iovita[2], Paul Chiroi[2], Antonia Harangus[2,3], Cristina Cismaru[4,5], Cornelia Braicu[2], Ioana Berindan-Neagoe[2]

1) County Hospital Cluj-Napoca, Romania

2) Research Center for Functional Genomics, Biomedicine and Translational Medicine, Iuliu Hatieganu University of Medicine and Pharmacy, Cluj -Napoca, Romania

3) Leon Daniello Pneumophthisiology Hospital, Cluj-Napoca, Romania

4) Clinical Hospital of Infectious Diseases of Cluj-Napoca, Cluj-Napoca, Romania

5) Department of Infectious Diseases and Epidemiology, Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania

Abstract

Background and aims. This study evaluates the potential of oxidative stress biomarkers, specifically glutathione disulfide (GSSG) and reduced glutathione (GSH), for differentiating bacterial and viral infections. Oxidative stress plays a crucial role in the immune response, and glutathione is a key regulator of cellular redox balance. The aim was to assess whether differences in GSH and GSSG levels could be used as diagnostic markers for infection type.

Methods. A chemiluminescence-based method evaluated GSH and GSSG as potential biomarkers for distinguishing between bacterial and viral infections. The GSH and GSSG concentrations were analyzed across bacterial, viral, and control groups.

Results. Our data revealed significant differences in the GSH/GSSG ratio between the analyzed groups, with bacterial infections showing higher oxidative stress markers compared to viral infections. A combined analysis of GSH and GSSG concentrations, visualized through heatmaps and ROC curves, improved diagnostic accuracy, with clustering patterns distinguishing infection types.

Conclusions. These findings suggest that the GSH/GSSG ratio could be used as a biomarker in distinguishing between bacterial and viral infections, offering potential clinical applications for more accurate diagnosis. Further research is required to validate these results in larger cohorts and to explore the underlying mechanisms of oxidative stress in pathogen-specific immune responses.

Keywords: glutathione disulfide, reduced glutathione, viral infection, bacterial infection, biomarker

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Address for correspondence:
Cornelia Braicu
braicucornelia@yahoo.com

Introduction

Infectious diseases are a significant public health problem worldwide, and early differentiation between bacterial and viral infections is essential for proper patient management [1-3]. Misdiagnosis can lead to inappropriate treatments, such as unnecessary antibiotic prescriptions for several illnesses, contributing to antibiotic resistance [4-6]. To improve diagnostic accuracy, healthcare providers can use advanced diagnostic tools such as polymerase chain reaction (PCR) and rapid antigen tests. These technologies can quickly and accurately identify the causal agent of an infection [7-10]. Additionally, investing in the continuous development of diagnostic techniques can further enhance the ability to distinguish between bacterial and viral infections. Antibiotic resistance can result in infections becoming more challenging to treat, leading to prolonged illness and increased mortality rates. It can also cause higher medical costs due to more extended hospital stays and the need for more

expensive alternative medications [5,6,11-13].

Furthermore, antibiotic resistance can lead to the spread of resistant bacteria, posing a significant threat to global public health. Implementing strict antibiotic stewardship programs in healthcare settings can help ensure that antibiotics are prescribed only when necessary and appropriate. Public awareness campaigns about the dangers of antibiotic misuse can educate the general population on the importance of following prescribed treatments and not demanding antibiotics for viral infections.

Additionally, global collaboration on surveillance and research can aid in tracking resistance patterns and developing new antibiotics and alternative treatments. The urgency of developing reliable biomarkers to differentiate bacterial from viral infections for clinical diagnosis cannot be overstated. Oxidative stress, which involves an imbalance between reactive oxygen species (ROS) and antioxidants, is a common feature of bacterial and viral infections. However, the degree and type of oxidative stress response may vary between infections. Using chemiluminescence assays, the oxidative stress response is associated with biomarkers such as glutathione disulfide (GSSG) and reduced glutathione (GSH). Therefore, these biomarkers can indicate the type of infection based on their levels during bacterial and viral infections.

The primary objective of this study was to investigate the diagnostic utility of GSSG and GSH as independent biomarkers for distinguishing bacterial from viral infections. We analyzed these biomarkers in bacterial, viral, and control samples to determine their discriminatory ability. We used statistical models such as ROC curve analysis and logistic regression to underscore the potential of GSH and GSSG as effective biomarkers for distinguishing bacterial and viral infections.

Methods

Sample collection from patients with bacterial and viral infections and healthy controls

This study included 48 patients, 20 with bacterial infection, 19 with viral infections, and 9 healthy controls (Table I). The inclusion criteria for our study required the presence of a viral or bacterial infection in patients with accessible plasma samples. We collected plasma from patients diagnosed with viral or bacterial infections, for whom informed consent was obtained, and control samples from the Biobank. The study received approval from the institutional Ethical Committees of both the Leon Daniello Pulmonology Hospital (Cluj-Napoca, Romania, no. 264/26.06.2018) and the Infectious Diseases Hospital (Cluj-Napoca, Romania, no. 15126/21.08.2024). Plasma samples were collected during hospitalization, frozen, and stored at −80° C. Clinical data were gathered for all participants, and informed consent was obtained before conducting any experimental evaluation in compliance with the ethical guidelines of the hospital and university.

GSH/GSSG-Glo evaluation using a chemiluminescence-based approach

This method offers high sensitivity and specificity, allowing for accurate measurements even at low concentrations. Additionally, it provides rapid results and is relatively easier to use than traditional methods. This makes it an ideal choice for high-throughput screening and various research applications. To perform the GSH/GSSG-GloTM Assay, prepare the samples and reagents according to the manufacturer's instructions. Next, the sample and reagents were added to a 96-well plate, followed by the addition of the luciferin generation reagent. Finally, the luminescence will be measured using a plate reader (Synergy Biotek), providing the glutathione levels in the samples.

Table I. List of the patients included in the study.

Sample	Infection	Sex	Age	GSH	GSSG	Sample	Infection	Sex	Age	GSH	GSSG	Sample	Infection	Sex	Age	GSH	GSSG
ZM1	Bacterial	F	88	y	y	ZM7	Viral	F	41	y	y	CTR16	Control	F	37	n	y
ZM2	Bacterial	F	42	y	y	ZM12	Viral	F	47	y	y	CTR18	Control	F	45	y	y
ZM8	Bacterial	F	33	y	y	ZM15	Viral	F	33	y	y	CTR19	Control	F	45	y	y
ZM13	Bacterial	F	45	y	y	ZM20	Viral	F	27	y	y	CTR20	Control	F	22	y	y
ZM16	Bacterial	F	22	y	y	ZM28	Viral	F	24	y	y	CTR25	Control	F	41	y	y
ZM19	Bacterial	F	27	y	y	ZM29	Viral	F	65	y	y	CTR23	Control	M	42	n	y
ZM21	Bacterial	F	22	y	y	ZM30	Viral	F	58	y	y	CTR16	Control	M	46	y	y
ZM22	Bacterial	F	38	y	y	ZM34	Viral	F	37	y	y	CTR18	Control	M	56	y	y
ZM24	Bacterial	F	38	y	y	DD28	Viral	F	42	y	n	CTR19	Control	M	19	n	y
ZM36	Bacterial	F	46	y	n	DD33	Viral	F	24	y	n						
ZM3	Bacterial	M	35	y	y	ZM6	Viral	F	33	y	y						
ZM10	Bacterial	M	42	y	y	ZM23	Viral	M	27	y	y						
ZM11	Bacterial	M	17	y	y	ZM25	Viral	M	34	y	y						
ZM18	Bacterial	M	31	y	y	ZM27	Viral	M	61	y	y						
ZM26	Bacterial	M	51	y	y	ZM32	Viral	M	27	y	y						
ZM31	Bacterial	M	34	y	y	ZM37	Viral	M	29	y	n						
ZM33	Bacterial	M	44	y	y	ZM38	Viral	M	25	y	n						
ZM9	Bacterial	M	29	y	y	ZM39	Viral	M	57	y	n						
ZM5	Bacterial	M	18	y	y	ZM17	Viral	M	33	n	y						

y - yes, n - no.

The GSH/GSSG-GloTM Assay provides a simple, rapid multiwell-plate format for determining GSH, GSSG, and GSH/GSSG ratios in cultured cells. The stable luminescent signals directly correlate with the sample's GSH or GSSG concentration, making the assay straightforward and efficient. Both GSH and GSSG determinations are based on the GSH-dependent conversion of a GSH probe in the presence of Luciferin-NT to luciferin by a glutathione S-transferase enzyme coupled to a firefly luciferase reaction. The luminescent signal is proportional to the amount of GSH, providing a quick and reliable measurement of glutathione levels.

Statistical analysis

Python was used as the primary software platform for the statistical analysis. The data were processed using the pandas' library for data organization and manipulation. In contrast, the scikit-learn library was employed to perform the Receiver Operating Characteristic (ROC) curve analysis and to calculate the Area under the Curve (AUC), which provides a quantitative measure of the ability of GSSG concentrations to differentiate between bacterial and viral infections. Additionally, logistic regression was performed using sci-kit-learn to assess the predictive power of GSSG as a biomarker for bacterial infections. The visualizations were generated using the matplotlib library, which was used to plot the sensitivity and specificity across various concentration thresholds—this combination of libraries and Python's analytical capabilities allowed for accurate statistical data evaluation.

Ingenuity Pathway Analysis (IPA) for Glutathione and Oxidized Glutathione, identification molecular networks

Considering the altered expression levels of glutathione oxidized and glutathione in plasma samples, these important molecules were integrated into Ingenuity Pathway Analysis (IPA) software, specifically designed to interpret complex biological data. IPA facilitated the investigation of biological functions and pathways linked to the redox state of glutathione by mapping these molecules to establish regulatory networks. This analysis provided insights into their roles in oxidative stress, detoxification, and immune response during bacterial and viral infections. The detailed output from IPA helped elucidate the molecular mechanisms contributing to the altered balance between glutathione and oxidized glutathione observed in the plasma samples.

Results

Evaluation of the GSH and GSSH using a chemiluminescence-based method

Our data were statistically analyzed using the chemiluminescence signal generated for GSH and GSSG to compare viral and bacterial infections in patients' serum samples. GSH and GSSH concentrations were explored as potential biomarkers for distinguishing bacterial and viral infections. The dataset was divided into three groups: bacterial infections, viral infections, and controls, as presented in figure 1. Analysis of GSH concentrations revealed specific patterns across the bacterial, viral, and control groups. On average, bacterial infections displayed higher GSH concentrations than viral infections, with controls showing intermediate levels. This rise in GSH levels in bacterial infections could indicate the discriminating oxidative response typically associated with bacterial pathogens. However, as reflected in the standard deviations, the variability within each group suggested that GSH levels alone do not provide a definitive separation between bacterial and viral infections.

The analysis for GSSG concentrations underscored distinct expressions across the bacterial, viral, and control groups. Bacterial infections generally showed the highest mean GSSG levels, which supports the concept that oxidative stress is more pronounced in bacterial-infected patients. This increase in GSSG levels likely reflects increased oxidative activity associated with bacterial-induced immune responses. The mean and median GSSG concentrations for bacterial samples were significantly higher than those for viral infections. In contrast, control samples displayed the lowest concentrations overall, as expected in non-infected individuals.

A **clustering heatmap** was also generated to illustrate how samples cluster based on their GSSG and GSH concentrations. As shown in figure 2, bacterial and viral infection samples group separate more distinctly when GSSG and GSH concentrations are considered together, compared to when each biomarker is used individually. The clustering patterns reveal that samples with higher GSSG levels tend to cluster in the bacterial group, while samples with higher GSH concentrations form distinct viral clusters. This visualization underscores the value of combining GSSG and GSH to capture more nuanced patterns in the data, which are not apparent when examining either biomarker alone.

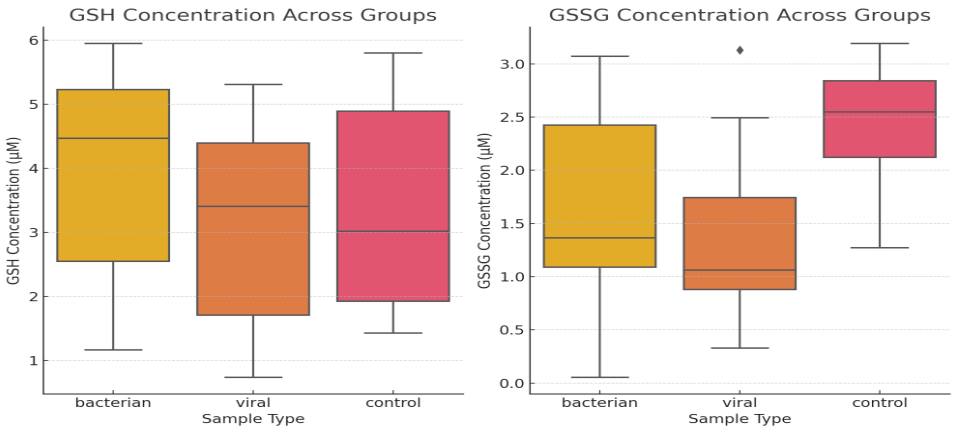

Figure 1. The expression level of GSH concentrations for Bacterial, Viral, and Control Groups using a chemiluminescence-based method. The rhomboid figure represents an outlier - outliers are points that fall outside the typical range of data. In this case, the outlier symbol indicates that the GSSG concentration for that particular sample falls outside the expected interquartile range (IQR).

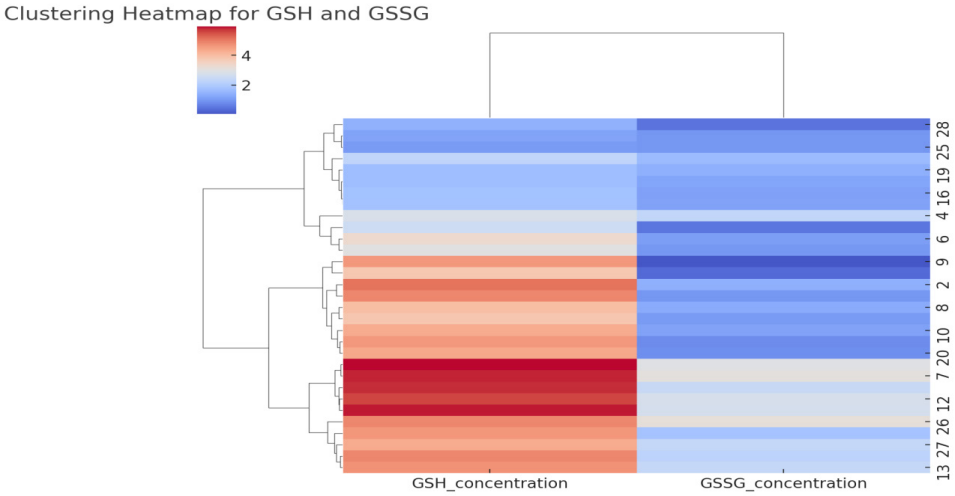

Figure 2. Clustering heatmap of samples based on combined GSSG + GSH concentrations.

Comparison of GSSG and GSH expression levels in the patients' cohort

The comparative analysis for GSSG and GSH reveals that both biomarkers exhibit elevated concentrations in bacterial infections compared to viral infections and controls. The increased levels of GSSG, a marker of oxidative stress, align with the immune system response during bacterial infections. Similarly, GSH, known for its role in cellular defense against oxidative damage, displayed a higher concentration in bacterial infections, suggesting a correlation between elevated GSH levels and bacterial infection-induced oxidative stress.

The clustering heatmap above (Figure 2) shows the distribution of **GSSG** and **GSH** concentrations for bacterial and viral samples. This visualization highlights the substantial overlap between bacterial and viral samples based on GSSG and GSH levels, illustrating that when these biomarkers are considered individually, there is no clear-cut distinction between bacterial and viral infections.

As the heatmap reveals, bacterial and viral samples cluster closely together, suggesting that neither GSSG nor GSH concentrations alone provide sufficient differentiation between the two infection types. This supports the conclusion that while these biomarkers may show trends associated with infection type, a more comprehensive, multivariate approach is likely required to improve diagnostic accuracy.

The ROC curve presented in figure 3 for the combined model approaches the top-left corner of the graph, representing high sensitivity and specificity. This suggests that integrating GSSG and GSH provides a more robust diagnostic tool for distinguishing bacterial from viral infections.

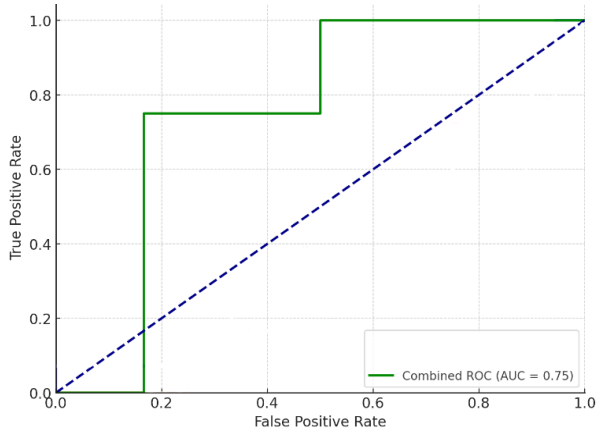

Figure 3. ROC curve for combined GSSG + GSH model in differentiating bacterial and viral infections.

Correlation Matrix of GSSG and GSH concentrations in viral and bacterial patients' cohort. A correlation heatmap (Figure 4) was generated to visualize the relationships between GSSG concentration, GSH concentration, and infection type (bacterial or viral). This heatmap displays the correlation coefficients, illustrating the extent to which these biomarkers are associated with bacterial and viral infections. The heatmap revealed a positive correlation between GSH concentration and bacterial infections (0.48), suggesting that higher GSH levels are associated with bacterial infections. On the other hand, the heatmap showed moderate negative correlations between GSH concentration and viral infections (-0.31), indicating lower GSH levels in viral infections. Interestingly, GSSG concentration exhibited a weak correlation with bacterial infections (0.02), while a moderate negative correlation was observed between GSSG concentration and viral infections (-0.29).

Ingenuity Pathway Analysis (IPA) for GSH and GSSG, identification molecular networks. IPA enabled a comprehensive investigation into the biological functions and pathways associated with the redox state of glutathione by mapping glutathione-related molecules to well-established metabolic and signaling networks. This approach allowed us to examine how the balance between GSH and GSSG is regulated, especially in oxidative stress, inflammation, and immune response during bacterial and viral infections. IPA highlighted key cellular detoxification, mitochondrial function, and redox signaling pathways by integrating these molecules into larger biological frameworks. These pathways are crucial for maintaining cellular homeostasis and protecting against oxidative damage caused by pathogens.

GSH and GSSG interact with various proteins and complexes (e.g., JAK1, FYN, EIF2AK1, MAPK8), enzymes related to detoxification (e.g., GSTM1, GSTT2, GSTP1, GSTA1) or chemical reagents and drugs that interact with the glutathione pathway (e.g., dithiothreitol, troglitazone, hydroxy amine), as observed in figure 5. All this is related to cellular and physiological functions like apoptosis and oxidative stress. This network provides an in-depth look at how glutathione is involved in different biochemical pathways and how it is regulated during oxidative stress and immune responses.

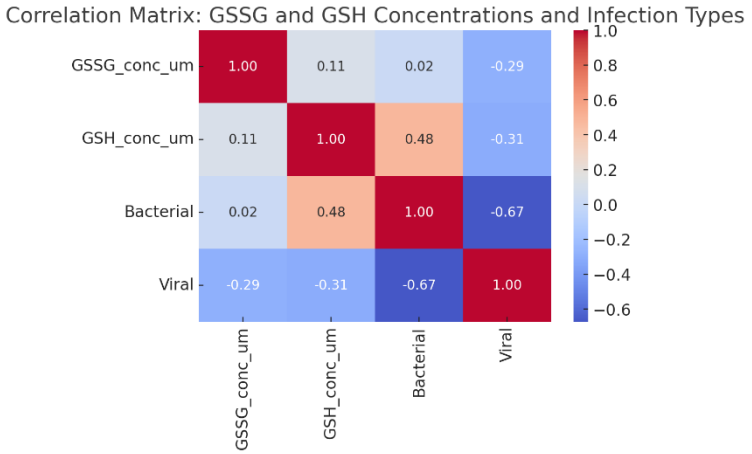

Figure 4 - Correlation Matrix of GSSG, GSH, in viral and bacterial patients' cohort.

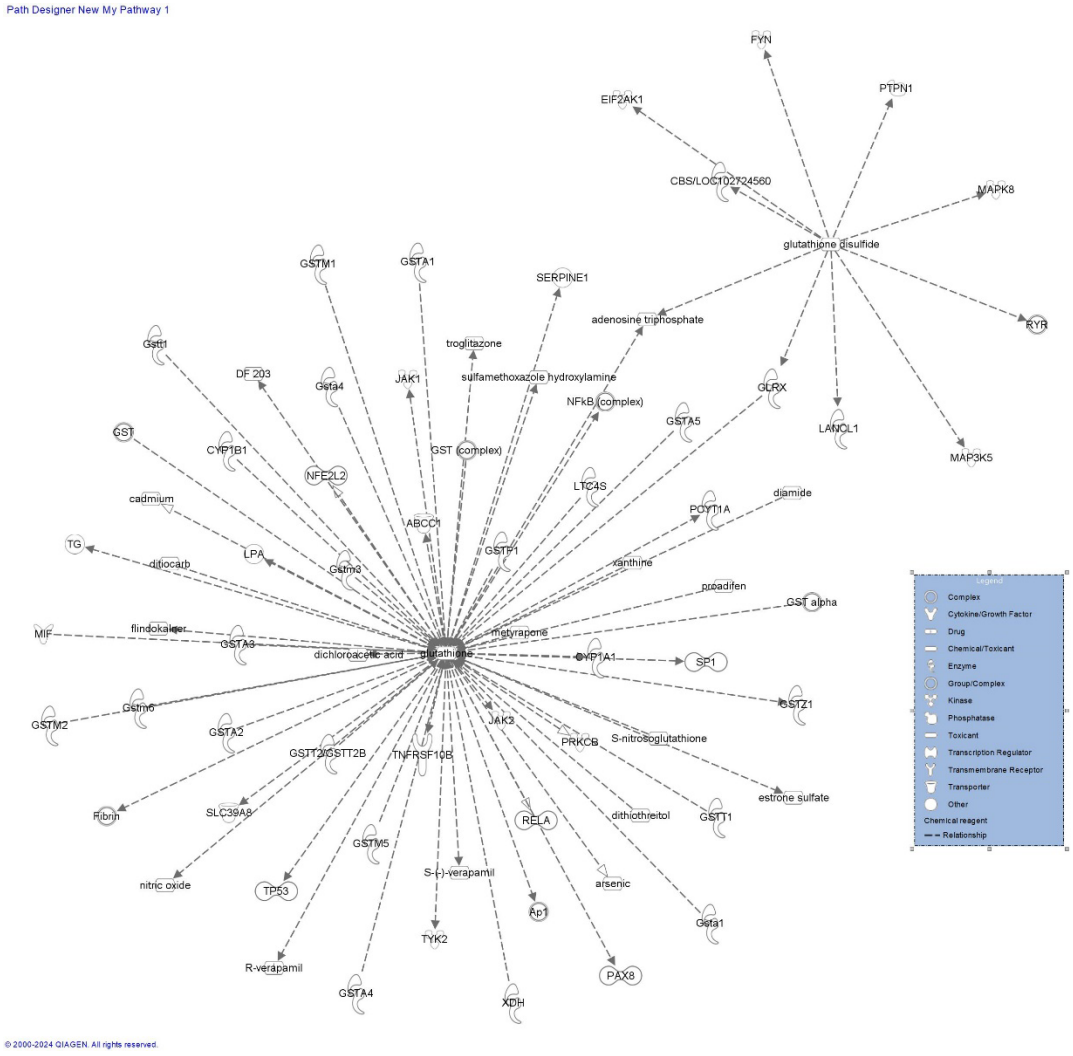

Figure 5. Ingenuity Pathway Analysis network showing the interconnected communication between oxidative stress biomarkers.

Discussion

Using a chemiluminescence assay, the current study evaluated the practicality of GSSG and GSH as potential biomarkers for differentiating bacteria from viral infections. Our results on patients' serum samples with bacterial and viral infections indicate that while these oxidative stress markers reflect the immune response during infection, their ability to discriminate between bacterial and viral infections is improved when used together rather than independently. GSSG and GSH are oxidative stress markers, and their levels are modulated during infection [14,15]. In our patients, bacterial infections demonstrated higher concentrations of GSSG and GSH than viral infections, in concordance with previous research suggesting that bacterial pathogens trigger a more intense oxidative burst in immune cells [16-18]. However, GSSG and GSH showed significant overlap between bacterial and viral infections. These findings suggest that while oxidative stress is elevated during infections, it is a general immune response not specific to bacterial pathogens. Chemiluminescence reflects the generation of ROS during the oxidative burst, and higher levels were observed in bacterial infections, supporting the hypothesis that these infections elicit more robust oxidative responses. The overlap in GSSG and GSH levels between bacterial and viral infections is likely due to the non-specific nature of oxidative stress. Both bacterial and viral infections trigger the production of ROS as part of the innate immune response. During the oxidative burst, ROS are released by immune cells, such as neutrophils and macrophages, to neutralize invading pathogens [19-21]. This process increases oxidative stress markers, regardless of whether the pathogen is bacterial or viral. While bacterial infections are associated with more pronounced oxidative responses, viral infections also

produce ROS through mechanisms such as the activation of interferon signaling pathways. Thus, while oxidative stress biomarkers provide insight into the immune system's activity, they do not offer pathogen-specific information. Age, gender and individual variability in immune responses contribute to the overlap between groups. Factors such as age, overall health, immune competency, and the severity of infection can influence oxidative stress levels. For example, patients with pre-existing conditions like diabetes or chronic inflammatory diseases may have elevated baseline oxidative stress, which could complicate the interpretation of GSSG and GSH measurements in the context of infection. Differentiating bacterial from viral infections remains a significant challenge in clinical diagnostics. While oxidative stress markers can indicate an active immune response, they lack the specificity to guide treatment decisions, such as whether to prescribe antibiotics. Inappropriate antibiotic use for viral infections is a significant driver of antibiotic resistance, emphasizing the need for more reliable diagnostic tools.

Furthermore, the IPA analysis shed light on specific molecular mechanisms, such as glutathione's role in neutralizing ROS, regulating the immune response, and modulating apoptosis. IPA also identified potential upstream regulators and downstream effects of altered glutathione levels, providing insights into how oxidative stress may contribute to the progression and severity of infections. The findings also revealed potential upstream regulators, such as cytokines and transcription factors, that influence the production and depletion of glutathione, further connecting these molecules to immune regulation and the pathophysiology of infections.

Conclusion

Our data showed the importance of immediately improving early detection kits and methods in point of care for infectious diseases. A global health problem, bacterial and viral infections continue to put pressure on the medical system worldwide, especially in low-income countries. The analysis evidenced that these two infection types could be identified using chemiluminescence assays, which can dissociate between bacterial and viral. Our study showed for the first time that simple chemiluminescence assays could used to detect bacterial and viral infections. Several limitations of this study should be noted. The relatively small sample size (20 bacterial, 19 viral, and nine control samples) limits the generalizability of the findings. A more comprehensive cohort of samples could improve the use of chemiluminescence assays to discriminate between bacterial and viral infections. While the study detected significant biomarker-level expression, larger sample sizes must confirm these results and provide a more comprehensive understanding of biomarker variability across different patient populations.

Further steps in this field must correlate cytokine expression and specific pathogen-associated molecular patterns to improve immediate diagnostic information. The findings from this study have several important clinical implications. Clinicians should be cautious in interpreting elevated oxidative stress markers, as these are indicative of immune activation but not specific to bacterial infections. Second, there is a need for more reliable diagnostic tools that can differentiate bacterial and viral infections, particularly in the context of antibiotic resistance. Developing point-of-care diagnostic devices that measure multiple biomarkers simultaneously could revolutionize infection diagnosis. Advances in biosensor technology and lab-on-a-chip platforms can potentially integrate oxidative stress markers with other relevant biomarkers, providing rapid, real-time diagnostic information in clinical settings. These technologies could help clinicians make more informed treatment decisions, improve patient outcomes, and reduce the inappropriate use of antibiotics.

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