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EDITORIAL

Is it enough to eliminate hepatitis C virus to reverse the damage caused by the infection?

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Abstract

Hepatitis C virus (HCV) infection represents one of the major causes of chronic liver disease, hepatocellular carcinoma and morbidity/mortality worldwide. It is also a major burden to the healthcare systems. A complete elimination of the HCV from the body through treatment is now possible. However, HCV not only alters the hepatic function. Several extra-hepatic manifestations are present in HCV-infected patients, which increase the mortality rate. Liver and gut are closely associated in what is called the "gut-liver axis". A disrupted gut barrier leads to an increase in bacterial translocation and an activation of the mucosal immune system and secretion of inflammatory mediators that plays a key role in the progression of liver disease towards decompensated cirrhosis in HCV-infected patients. In addition, both qualitative and quantitative changes in the composition of the gut microbiota (GM) and states of chronic inflammation have been observed in patients with cirrhosis. Thus, a successful treatment of HCV infection should be also accompanied by a complete restoration of GM composition in order to avoid activation of the mucosal immune system, persistent inflammation and the development of long-term complications. Evaluation of GM composition after treatment could be of interest as a reliable indicator of the total or partial cure of these patients. However, studies focused on microbiota composition after HCV eradication from the body are lacking, which opens unique opportunities to deeply explore and investigate this exciting field.

Key words: Hepatitis C infection; Inflammation; Virus eradication; Direct-acting antivirals; Gut microbiota

Core tip: Hepatitis C infection represents one of the major causes of chronic liver disease, hepatocellular carcinoma and morbidity/mortality worldwide. A complete elimination of the hepatitis C virus (HCV) from the body through treatment is now possible. However, HCV not only alters the hepatic function. In fact, changes in gut microbiota composition (GM) and gut barrier that leads to an increased bacterial translocation and inflammation have also been observed. Thus, a successful treatment

of HCV infection should be accompanied by a complete restoration of GM and inflammation. Studies focused on GM after HCV eradication are lacking, which opens unique opportunities to deeply explore this exciting field.

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Infection with hepatitis C virus (HCV) is one of the major causes of liver damage and morbidity/mortality worldwide[1]. The spectrum of this disease is quite variable, ranging from acute hepatitis to cirrhosis and hepatocellular carcinoma (HCC). In fact, HCV is considered the most important risk factor for the development of this type of cancer[2], one of the more common cancers in the general population that has substantially increased in recent years.

HCV infection is a major burden to the healthcare systems, as it is the most frequent indication for virus-related liver transplantation in the western world[3]. In addition, patients diagnosed with HCV showed increased morbidity, with higher hospital admission rates[4] and with mortality rates three times higher than that of the general population[5]. In a recent meta-analysis, the number of people with anti-HCV antibodies has been estimated at 185 million in 2005 (2.8% of the human population), with an estimation of 130-170 million people chronically infected[6]. Overall, between 300000-700000 people die every year due to liver diseases associated with HCV-infection[7,8].

Liver is, by far, the most affected organ, but HCV infection is definitely not a liver-limited disease. Actually, HCV infection has been associated with other extra-hepatic manifestations that include thyroid diseases, renal and cardiovascular diseases, eye and skin diseases, lymphomas, mixed cryoglobulinemia, dyslipidemia, diabetes and central nervous system diseases (brilliantly reviewed by several authors)[9-13]. In fact, up to 74% of HCV-infected patients experienced some form of these extra-hepatic manifestations[14]. Therefore, HCV infection showed a higher mortality rate due to the presence of these extra-hepatic complications[15-17]. Several studies have also suggested that HCV may infect other tissues apart from liver. Thus, HCV has been found in peripheral blood mononuclear cell[18,19], kidney, heart, pancreas, and in intestine[20,21]. The infected extra-hepatic tissues might act as potential reservoirs for HCV, and could play a role in both HCV persistence and reactivation of infection but could also contribute to the aforementioned extra-hepatic manifestations associated with HCV infection. Despite the fact that a growing interest has recently emerged concerning the extra-hepatic manifestations of chronic HCV infection, as demonstrated by the increasing number of reviews recently published, there is no scientific evidence that could demonstrate an association between the presence of the virus in other tissues different from liver and the extra-hepatic complications. Therefore, this issue deserves further investigation.

One area of investigation that has been the focus of much recent interest in the last years is the role of intestinal microbiota in health and disease[22]. Microbiota is defined as the collective microbial community inhabiting a specific environment, including bacteria, archaea, viruses, and some unicellular eukaryotes. Microbiota, its evolutive dynamics and influence on host through its protective, trophic and metabolic actions, has a key role in health and opens unique opportunities for the identification of new markers of the physiopathological state of each individual. Recent studies have demonstrated that changes in gut microbiota (GM) contribute to an increased intestinal permeability and, consequently, increased bacterial translocation and endotoxemia, which triggers inflammation and several deleterious actions[23]. In this sense, changes in GM composition is associated with plenty disorders, including liver disorders[22,24-27].

The effects of GM are not limited to the intestine (gut). Indeed, the gut and the liver are closely associated and there is continuous bidirectional communication between these two organs through the bile, hormones and other products of digestion and absorption. This association is known as the "gut-liver axis" and includes transfer of molecules associated with the gut microbiome to the liver and on the other way round[24,28]. Therefore, it is plausible that the composition of the intestinal microbiota could have direct and indirect effects on the function and physiology of the liver and possibly liver disease progression[29-31]. In addition, it has also been suggested that several liver products, such as bile acids, could directly influence the GM composition[30].

A disrupted gut barrier leads to an increase in bacterial translocation and to an activation of the mucosal immune system and secretion of inflammatory mediators that has a key role in the development of several liver disorders associated with HCV-infection, especially in the progression of liver disease towards decompensated cirrhosis in both HCV-mono infected and HCV/HIV co-infected patients[32-36]. In this context, the study carried out by Sandler *et al*[37] (2011) in HCV-infected patients showed that LPS-induced activation of both circulating monocytes and resident Kupffer cells was associated with severe hepatic fibrosis and failure to respond to therapy (based on interferon or pegylated interferon with or without ribavirin) and predicts progression to end-stage liver disease independent of the degree of fibrosis. In addition, several studies have demonstrated both qualitative and quantitative changes in the composition of the GM in patients with cirrhosis (summarized by Betrapally *et al*[38]). More specifically, the alteration in GM of cirrhotic patients (with and without HCV infection) is characterized by an overgrowth of potentially pathogenic bacteria (*i.e.,* gram negative species) and a decrease in autochthonous familiae[24]. Significant differences in the microbiota community and metabolic potential have

also been detected in the fecal microbiota of patients with hepatitis B liver cirrhosis[39]. Therefore, preservation of GM composition - through the usage of different approaches such as probiotics, prebiotics, *etc.,* arises as a promising tool to prevent and/or to treat the development of these liver disorders[40-44]. However, studies focused on microbiota composition of a large population of HCV patients (over the entire disease spectrum) are lacking and only studies concerning cirrhosis and HCC independently of their etiology can be found.

On the other hand, it is important to mention that one of the main objectives of health professionals when treating infectious diseases is to eliminate the pathogenic microorganism responsible for such disorder. To achieve this, physicians are provided with a large arsenal of antibiotics, antivirals, antiretroviral, *etc*., that have appeared in the last decades thanks to the spectacular progress of scientific research. In the context of HCV-infection, the last five years have been crucial in the fight against this infection. A few years ago, physicians only had therapies based on the combination of weekly pegylated interferon-α and daily doses of ribavirin to treat this infection. The efficacy of these therapies was not higher than 50%, and their mechanism of action was not direct against the virus, but was based on enhancing the immune system. In 2011, the arrival of first-generation direct-acting antivirals has shown successful rates of virus elimination from the body in more than 75% of the cases. Unlike the previous therapies, these new regimens cause fewer side effects, they do not require monitoring and most of them are pangenotypic. These therapies are also simpler and require a shorter duration. Thus, and despite the fact that there is not an effective vaccine yet, this could be the beginning of the end of hepatitis C disease[1,45]. However, a complete cure of HCV-infection requires not only the elimination of the virus from the body, but also would imply an improvement in liver and in the extra-hepatic manifestations. In this sense, the study from Innes *et al*[46] (2015), demonstrated a clear association between achievement of HCV cure [as evidenced by sustained viral response (SVR)] and a decrease in both liver-related and all-cause mortality. A large study of HCV-infected patients in the Veteran's Administration database also demonstrated that non-liver-related mortality was significantly reduced among patients who achieved SVR who had comorbidities that included coronary artery disease, diabetes, and hypertension. It was suggested that decreased chronic inflammation associated with HCV was a key factor in mortality decline[47,48]. It is important to highlight that changes in microbiota composition are present in states of chronic inflammation. Thus, a successful treatment of HCV infection should be also accompanied by a complete restoration of GM composition in order to avoid activation of the mucosal immune system, persistent inflammation and the development of different long-term complications. Thus, evaluation of GM composition after treatment could be of interest as a reliable indicator of the total or partial cure of these patients. Only a very preliminary study has been recently published in this regard[49]. It demonstrated that the pro-inflammatory state and the changes observed in GM composition of HCV-infected patients with cirrhosis were not improved regardless of at least one year of SVR. This persistent dysbiosis could contribute towards varying rates of improvement after HCV eradication in cirrhosis. However, what happen in HCV-infected patients with a lower degree of fibrosis/liver damage? Could HCV have a direct effect on GM composition since the presence of this virus has been demonstrated in intestine? or the changes observed in GM are only secondary to liver damage induced by the virus? These are only a few questions that arise in this exciting field and that deserve further investigation. A deep evaluation of the short, medium and long-term consequences of the new HCV treatments is needed, specially focused on the effects on GM composition, bacterial translocation and inflammation.

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MINIREVIEWS

Platelet indices in neonatal sepsis: A review

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Abstract

Thrombocytopenia is a common hematological abnormality in neonates with sepsis. The autoanalyzers now-a-days readily provide platelet indices along with platelet counts without any additional cost. However these indices are not given proper weightage often. The important platelet indices available for clinical utility include mean platelet volume (MPV), platelet distribution width and plateletcrit that are related to morphology and proliferation kinetics of platelets. Studies in adult patients reported their role in the diagnosis of severe sepsis and prognosis of adverse clinical outcomes including mortality. Abnormal MPV can aid diagnosing the cause of thrombocytopenia. Low MPV associated with thrombocytopenia has been found to result in clinical bleeding. Other indices, however, are less studied. The studies addressing the importance of these platelet indices in neonatal sepsis are limited. The current review gives an overview of potential utility of important platelet indices in neonatal sepsis.

Key words: Sepsis; Platelet indices; Thrombocytopenia; Bleeding; Neonate

Core tip: Sepsis in neonates often results in thrombocytopenia and changes in platelet indices. The important platelet indices such as mean platelet volume (MPV), platelet distribution width and plateletcrit are related to morphology and proliferation kinetics of platelets. All these indices are readily available with no additional cost while performing routine blood counts using autoanalyzers. Studies in adult patients reported the potential role of platelet indices in the diagnosis of severe sepsis and prognosis of adverse clinical outcomes including mortality. Abnormal MPV can aid diagnosing the cause of thrombocytopenia. Low MPV associated with thrombocytopenia has been found to result in clinical bleeding. The current review gives an overview of potential utility of important platelet indices in neonatal sepsis.

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INTRODUCTION

Neonatal sepsis is often accompanied by thrombocytopenia and late onset sepsis remains an important cause of thrombocytopenia in neonates[1-5]. Although important platelet indices are readily available while obtaining routine complete blood counts (CBC), they are less studied. The platelet indices have gained more importance in the recent studies. Among many platelet indices, the indices related to morphology and platelet kinetics such as mean platelet volume (MPV), platelet volume distribution width (PDW) and plateletcrit (PCT) are studied in sepsis. The role of platelet indices in sepsis has been reported in adult studies. Such studies reported their role in the diagnosis of sepsis and severe sepsis[6,7]. In addition, these indices have been found to be useful in the prognosis of adverse clinical outcomes including mortality[6-8]. Guclu *et al*[6] reported that MPV and PDW were significantly different between sepsis patients and control group. They concluded that patients having PDW greater than 18% have higher risk for death. Gao *et al*[7] reported usefulness of MPV in predicting adverse outcome in septic shock patients. Usefulness of continuous monitoring of MPV and thereby identifying the change in MPV 72 h after admission in stratifying mortality risk in patients with severe sepsis and/or septic shock was reported by Kim *et al*[8]. Furthermore, Becchi *et al*[9] reported the usefulness of MPV trend in sepsis patients along with platelet count.

A combination of increased destruction and inadequate production of platelets during sepsis-induced thrombocytopenia of the neonate may result in release of young platelets into the circulation. An increased proportion of young platelets may result in increased MPV. A significant increase in MPV from baseline values in neonatal sepsis has been reported by Guida *et al*[2]. O'Connor *et al*[3] described changes of MPV in neonates with Coagulase negative *Staphylococcus* sepsis. In the subsequent sections, the changes of platelet indices during neonatal sepsis and clinical utility of three important indices have been discussed.

PLATELET INDICES

CBC tests with automated hematology analyzers are one of the most commonly ordered tests during neonatal sepsis work up. These analyzers rapidly measure the platelet count and also the platelet indices. Platelet indices are biomarkers of platelet activation. These indices are of diagnostic and prognostic value without any added costs in a variety of settings including sepsis. In automatic CBC profiles, MPV, PDW and PCT are a group of platelet indices determined together. These indices are related to morphology and proliferation kinetics of platelets and hence have a definite clinical utility in patients with sepsis. The other indices include mean platelet component, mean platelet mass, platelet component distribution width, platelet large cell ratio (P-LCR) and immature platelet fraction (IPF). The latter indices are studied very rarely. P-LCR often correlates to MPV but is more sensitive to changes in platelet size[7]. The IPF rises in patients with peripheral consumption or destruction of platelets. It is normal or low in patients with marrow failure.

The MPV is the arithmetic mean volume of the platelets derived from the platelet histogram on automated Coulter counters. It is expressed in femtoliters (fL). The platelet volume is regulated by cytokine dependent megakaryocyte ploidy and platelet number[10,11]. In the settings of decreased platelet production such as sepsis, young platelets that are bigger and more active enter the circulation and hence MPV levels increase. Increased MPV indicates increased platelet diameter. Therefore, increased MPV is useful clinically as a marker of production rate and platelet activation. The average MPV is 7.2–11.7 fL in healthy human subjects. The paucity of gestational age-based normative data has limited the clinical utility of MPVs in neonatal medicine. Wiedmeier *et al*[12] reported that MPVs are rather constant from 22 to 42 wk of gestation with a slight but statistically significant decrease between the earlier vs later gestations. They also provided 5th and 95th centile for MPV for different gestations.

PDW is an indicator of volume variability in platelets size and reflects the heterogeneity in platelet morphology[10,11]. It increases when there is platelet anisocytosis. The PDW reference intervals range from 8.3% to 56.6%. Under physiological conditions, there is a direct relationship between MPV and PDW; both usually change in the same direction.

PCT is the volume occupied by platelets in the blood as a percentage and calculated according to the formula, PCT = platelet count × MPV/10000. Under physiological conditions, the amount of platelets in the blood is maintained in an equilibrium state by regeneration and elimination. The normal range for PCT is 0.22%-0.24%.

MECHANISMS OF THROMBOCYTOPENIA AND ALTERATIONS IN PLATELET INDICES DURING SEPSIS

Because thrombocytopenia is a commonly encountered hematologic complication in neonates with sepsis, the mechanisms for thrombocytopenia have been explored. The measurement of circulating megakaryocyte precursors provides a good indicator of megakaryocytopoiesis, and hence platelet production in neonatal sepsis[13]. Thrombopoietin (Tpo) is the principal physiologic regulator of megakariocytopoiesis and platelet production. The circulating Tpo levels were found to be high in the face of low platelet counts in neonates with sepsis[14]. Immune cells recognize pathogens through Toll-like Receptors (TLRs). The TLRs allow platelets to recognize bacterial proteins during sepsis and regulate platelet immunity and function[15]. Two TLRs, TLR2 and TLR4, have been shown to augment platelet activation and alter its function from hemostatic regulator to immune sentinel. Furthermore,

septic neonates up-regulate Tpo production, leading to increased megakaryocytopoiesis and platelet release[16]. As platelet indices are biomarkers of platelet activation, in the settings of sepsis, these indices also change accordingly.

MPV

Among platelet indices MPV is the most commonly studied platelet index in neonatal sepsis. During conditions of rapid platelet turnover, increased MPV signifies the release of larger, younger platelets into the circulation. Although MPV varies with gestational age and chronologic age, construction of rigorous normal curves for values of the MPV is difficult in premature infants. Wiedmeier *et al*[12] found MPVs being rather constant from 22 to 42 wk of gestation. However, it is wiser to obtain the baseline values of MPV for comparison with subsequent values during neonatal sepsis.

A statistically significant increase in MPV with neonatal sepsis from baseline values (mean change in MPV 0.30 femtoliters; 95%CI: 0.12–0.47) was reported by Guida *et al*[2]. They reported this abnormality while studying platelet counts in 154 blood culture proven neonatal sepsis. The study involved Gram negative, Gram positive and fungal infections in neonates. They did not observe any organism-specific changes in MPV. O'Connor *et al*[3] reported increased MPV during coagulase-negative Staphylococcal sepsis in neonates even though platelet counts were normal.

The relationship between platelet count (PC) and MPV was studied by Becchi *et al*[9]. The results were expressed as means and frequency distributions. They reported a negative correlation (95%CI; r = -0.34; P < 0.0001) between PC and MPV with an inverse trend during sepsis course.

Catal *et al*[17] found a positive correlation between MPV and other inflammatory markers, IL-6 and CRP in neonatal sepsis. A MPV value of 10.35 fL was identified as the cut off value in patients probably resulting in sepsis with a sensitivity of 97.8% and specificity of 78.7% (AUC = 0.949; P < 0.001), and a MPV value of 10.75 fL was determined as the cut off value at diagnosis in patients possibly resulting in death with a sensitivity of 95.2% and a specificity of 84.9% (AUC = 0.944; P < 0.001).

Mitsiakos *et al*[18] reported that platelet mass levels could play an important role in predicting the occurrence of intracranial hemorrhage in neonates with sepsis.

The effects of different infectious agents on platelet count and indices in neonatal sepsis were studied by Akarsu *et al*[19]. They studied these values at baseline and at least 10 d after the onset sepsis. A MPV of > 9.5 fL and PDW of > 16.8 were considered high. Of 86 sepsis episodes involving Gram negative and Gram positive bacteria, 39.5% were found to be associated with thrombocytopenia, 13.9% with an elevation in baseline MPV and PDW, 11.6% with an elevation in baseline MPV and 72.1% with an elevation in baseline PDW. Neonates with MPV over 10.8 fL and/or PDW over 19.1 were found to have significantly increased bacteremia. Although there was an increase in MPV and PDW from baseline, there were no differences between different organism groups.

An understanding of the pathophysiology of alterations in platelet volume and the inverse relationship between platelet volume and count hence is a prerequisite for the successful clinical application of platelet volume measurements[20].

PLATELET DISTRIBUTION WIDTH

Farias *et al*[21] reported the PDW median of 13.3% with a reference range of 10.0%-17.9% for the 5th-95th percentiles with a confidence interval of 95% for normal individuals. Akarsu *et al*[19] addressed PDW changes in neonatal sepsis. By considering PDW of > 16.8 as high, they found an elevated baseline PDW in 72.1% of neonates with sepsis. However they did not find any organism specific response in PDW. Catal *et al*[17] reported higher levels of PDW along with higher MPV during sepsis episodes on consecutive days among non-survivors.

PLATELETCRIT

Of the several platelet indices PCT is studied less often in neonatal sepsis. The variation in MPV affects PCT. There is a significant overlap of PCT between thrombocytopenic patients and patients with normal platelet counts. Role of platelet mass in predicting the occurrence of intracranial hemorrhage in neonates with sepsis has been reported by Mitsiakos *et al*[18].

LIMITATIONS IN CLINICAL UTILITY OF PLATELET INDICES

Platelet volumes are frequently measured in blood samples collected in ethylenediaminetetraacetic acid (EDTA). Factors affecting platelet counting such as interference from cells or cell fragments, inadequate detection of large platelets or platelet clumps also influence platelet indices that are calculated from the platelet distribution curve[22]. An overestimation of MPV, a higher PDW and an increase in fraction of large cells may occur if red blood cells are misclassified as platelets. In severe thrombocytopenia, difficulties in obtaining a sufficient platelet distribution curve may limit the calculation of other platelet indices. Concerns have been raised about the recommended anticoagulant for platelet counting, K2 or K3 EDTA, because it affects MPV. Transmission electron microscopy findings suggested more activation of platelets in EDTA samples[23]. ACD/Na2EDTA has been suggested as an ideal anticoagulant for the study of MPV because it inhibits platelet activation while maintaining the platelets in their normal discoid shape[24]. The methods of measurement of MPV are also important. EDTA causes an increase in MPV from 7.9% within 30 min to 13.4% over 24 h when measured by impedance and decreases by 10% when determined by an optical method. Because

time delay is likely to affect PDW and other indices sample needs to be processed within 120 min. Pseudo-thrombocytopenia due to agglutination of platelets caused by EDTA should also to be kept in mind[25].

MPV, PDW and PCT are not only altered in sepsis but also in other neonatal pathological conditions[26-29]. This fact further complicates the clinical utility of platelet indices during neonatal sepsis. Gestational age, prematurity and birth asphyxia having some influence on these indices has been reported by Kannar *et al*[26]. Premature neonates with sepsis may have other comorbidities such as bronchopulmonary dysplasia (BPD) and intraventricular hemorrhage (IVH). Higher MPV level was noted in BPD and IVH groups in a study by Bolouki Moghaddam *et al*[27].

A decreased platelet count and PCT, an increased PDW and no difference in MPV among preterm neonates have been reported by Wasiluk *et al*[28] while studying samples from umbilical arterial blood. The large platelet count (LPLT) was found to be diminished in preterm neonates (5.23%) in comparison with term neonates (6.12%). They also reported higher MPV, lower LPLT and lower PCT among small for gestation neonates. Higher PDW, lower PCT and higher but not statistically significant MPV in preterm neonates compared to term neonates were reported by Sandeep *et al*[29].

CONCLUSION

Sepsis in neonates often results in thrombocytopenia and changes in platelet indices. The important platelet indices available for clinical utility include MPV, PDW and PCT. All these indices are readily available with no additional cost while performing routine blood counts using autoanalyzers. Platelet indices are helpful in the diagnosis as well as follow-up of sepsis including assessing the response of antimicrobial treatment if interpreted cautiously. High MPV and PDW have a high specificity for the identification of bacteremia and have a good predictive value. Neonatal studies support their clinical application but limitations should be kept in mind while interpreting results.

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REVIEW

Management of periprosthetic infections

Félix Vilchez-Cavazos, Gregorio Villarreal-Villarreal, Victor Peña-Martinez, Carlos Acosta-Olivo

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Abstract

Periprosthetic joint infection (PJI) is considered one of the most challenging complications compromising patient health and is considered an economic burden. Despite all strategies PJI prevalence is between 1%-2%. Considerable efforts have been investigated in the past decade to diminish or erradicate PJI prevalence. This article manages the definition of PJI and the new major and minor criteria from Parvizi *et al* Then a scientific analysis of every minor and major criteria. Multidisciplinary management is reccommended according to guidelines. A numerous of surgical options exist each and everyone with its indications, contraindications and specific antibiotic therapy regimen. Surgical options are: (1) irrigation and cleaning with retention of the prosthesis with a success rate 0%-89%; (2) single-stage revision surgery with a succes rate of > 80%; and (3) two-stage revision surgery (authors preferred method) with a succes rate of 87%. Radical treatment options like arthrodesis and amputation are reserved for specific group of patients, with a succes rate varying from 60%-100%. The future of PJI is focused on improving the diagnostic tools and to combat biofilm. The cornerstone of management consists in a rapid diagnosis and specific therapy. This article presents the most current diagnostic and treatment criteria as well as the different surgical treatment options depending on the type of infection, bacterial virulence and patient comorbidities.

Key words: Periprosthesic joint infection; Arthroplasty; Arthrocentesis; Infection; Diagnosis; *Staphylococcus aureus*

Core tip: The total replacement surgery is a highly effective surgery that improves the quality of life of patients. The periprosthetic infection is considered a devastating complication that increases patients morbidity, mortality and an economic burden. The cornerstone of management consists in a rapid diagnosis and specific therapy. This article presents the most current diagnostic and treatment criteria, as well as the different surgical treatment options depending on the type of infection, bacterial virulence and patient comorbidities.

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INTRODUCTION

Total joint replacement is a highly effective surgery that provides relief of pain, improves the range of motion, independence, and lastly, quality of life in the patient[1]. It is estimated that in 2030 a total of 4 million total hip and/or knee replacements will be done every year in the United States[2]. Prosthetic infections are considered a serious and devastating complication of total replacement; in general, the incidence of this complication is 1%-2%[3,4]. Nonetheless, there are reports ranging from 0.3% by the British Medical Research Council[5] up until 7%-16% in hip revision surgeries according to the Scandinavian Arthroplasty Report[6].

The key for the management of a prosthetic infection is based on an early diagnosis, which will allow adequate and fast treatment[7]. However, this represents a clinical burden, since the majority of the cases we are up against are complex, immunocompromised patients and antibiotic-resistant bacteria[8]. It also represents an economical burden since a prosthetic infection increments costs by 76% and 52% in total hip replacement and total knee replacement surgeries, respectively[9].

The objective of the present article is to update and summarize the diagnostic and therapeutic methods in periprosthetic joint infections (PJIs) in both knee and hip arthroplasty.

DIAGNOSIS AND DEFINITION OF PROSTHETIC INFECTION

For the diagnosis of prosthetic infection a high suspicion and laboratory studies are needed. There is no gold standard for the diagnosis of prosthetic infections, rather a series of clinical findings, laboratory and imaging studies guide the diagnosis[8]. In 2011, the Musculoskeletal Infection Society proposed a series of major and minor criteria[10], the latter then modified by the International Consensus Meeting on PJIs to give a numeric value to the serological markers[11].

To consider the diagnosis a prosthetic infection, one of the following criteria must be met: (1) two positive periprosthetic cultures (fluid or tissue) for the same microorganism; (2) the presence of sinus tract that communicates with the joint; and (3) three of the following criteria exist: Increase of 100 mg/L of C-reactive protein (CRP) in an acute infection; > 10 mg/L in a chronic infection and a rise in the erythrocyte sedimentation rate (ESR) > 30 mm/h in a chronic infection (not applicable in acute infections); elevated synovial leukocyte count (> 10000 cells/μL in acute and > 3000 cells/μL in chronic infections) and/or ++ or more in Leukocyte esterase dipstick test; elevated synovial neutrophil percentage (PMN%); > 90% in acute and > 80% in chronic infections; positive preriprosthetic histological analysis (> 5 neutrophils per field); a single positive culture (fluid or tissue).

ALGORITHM FOR THE APPROACH TO THE DIAGNOSIS OF PERIPROSTHETIC INFECTION

To achieve a systematic approach to diagnosing periprosthetic infections, in 2010 Della Valle *et al*[12] proposed an algorithm in the American Academy of Orthopedic Surgeons (Figure 1); changes have been made to this algorithm, such as the proposal of Parvizi *et al*[13] in 2016. However, in all cases this algorithm is only a tool and should never be considered a diagnosis. Any case of high clinical suspicion of infection should be subjected to this algorithm[11].

RISK FACTORS, HISTORY AND CLINICAL PRESENTATION

There are predisposing factors such as systemic malignancy, diabetes mellitus, rheumatoid arthritis, immunocompromised host, obesity, malnutrition, intravenous drug use, steroid therapy, systemic skin diseases, history of prior total replacement, and previous history of septic arthritis; intraoperative factors such as low body temperature, hypoxemia, duration of surgery, contaminated implants, and flow and configuration of the operating room; postoperative factors such as hematoma formation, transfusions, Foley catheter > 24 h as well as surgical site infection[14-16]. Clinically, patients with prosthetic infection usually present with pain, wound dehiscence and wound output[8]. However this varies significantly according to the evolution time and the pathogen involved[13]. Patients with less than 3 mo of evolution present with pain and rapidly progressive stiffness. On physical examination edema, erythema, warmth, increased sensibility and/or fever, effusion, surgical site infection and wound edge necrosis are usually present. Patients with 3-12 mo evolution usually present pain and slow but progressive stiffness. They are usually indistinguishable from aseptic loosening or present with an active fistula into the joint. In infections of > 12 mo of evolution the patient can present symptoms in two ways: (1) acute onset of pain and stiffness with a history of trauma or bacteremia (acute hematogenous infection); and (2) chronic pain and stiffness. The patients with acute hematogenous infections, clinically presents with more severe symptoms of pain, redness, warmth, increased tenderness and/or fever compared with patients with acute infections[13,17,18]. An important sign is fever, although it is considered a cardinal symptom of infection, it is reported that there may be an increase in the temperature of the

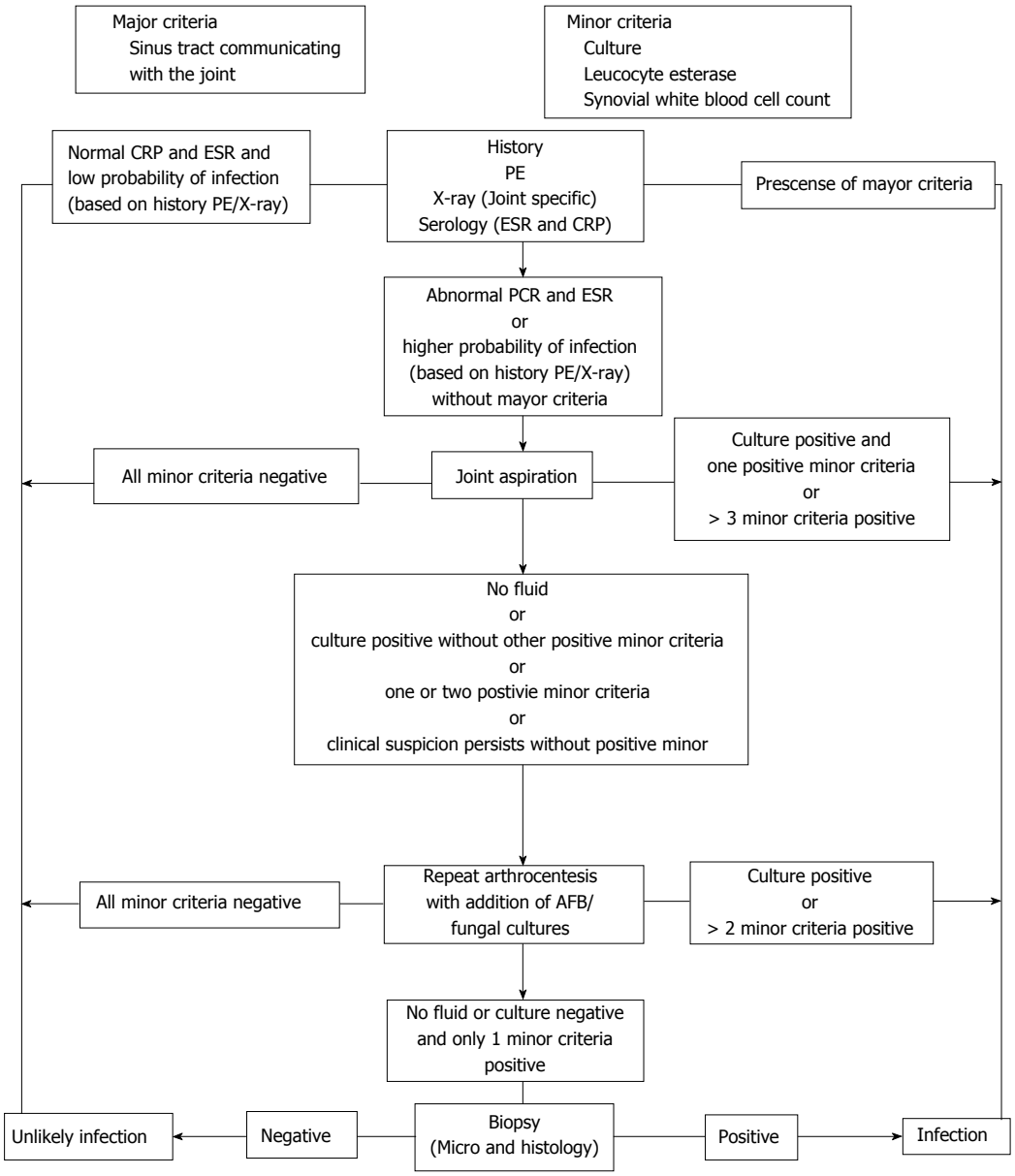

Figure 1 Simplified algorithm for approaching a patient with a probable periprosthesic joint infection, proposed by Della Valle *et al*[12]. CPR: C-reactive protein; ESR: Erythrocyte sedimentation rate; PE: Physical examination.

postoperative patient of a total replacement surgery for up to five days and is considered as a physiological postsurgical process[19].

IMAGING STUDIES

Because of their ease, fast delivery, and low cost, plain radiographs are the study of choice even if they have low sensitivity and specificity for the diagnosis of a prosthetic infection[13,20]. In regards to other studies, the evidence doesn't show a routine use. For example magnetic resonance imaging, produces visual artifacts, is difficult to interpret and has a high cost. Ultrasound is limited to the acquisition of collections and is operator-dependent[20-22]. Except for plain radiographs, none of the aforementioned studies are part of the current recommendations for the management of prosthetic infection. The radiographic findings are easy to interpret and amongst them are: (1) focal osteolysis (radiolucency > 2 mm in the bone-metal interface or cement-bone); (2) loosening of the components; (3) cement fractures; and (4) subperiosteal reaction[20,23]. Regarding gammagraphy, there is no consensus for the use in diagnosis of periprosthetic infection; even the American Academy guidelines do not recommend its routine use[12].

SEROLOGICAL STUDIES: BLOOD COUNT, CRP AND ESR

In the current diagnostic criteria for infection, CRP and ESR are part of the minor criteria for prosthetic infection diagnosis and are studies every patient with high suspicion for prosthetic infection should undergo[11]. These markers can be elevated in patients with rheumatic or chronic inflammatory diseases. It is reported that an

ESR > 30 mm per hour has a sensitivity of 82% and 85% specificity, a positive predictive value of 58% and a 95% negative predictive value. Meanwhile, CRP > 10 mg/L is associated with 96% and 92% sensitivity and specificity respectively; with a positive predictive value of 74% and a negative predictive value of 99%[24]. Another advantage offered by the CRP over the ESR is the return to normal values in 3 wk compared with ESR which can take up to a year[25,26]. The current recommendation is that all patients with suspected prosthetic infection undergo both serological studies, as the combination of these normal parameters is an excellent predictor of absence of infection and the combination of both positive tests approaches a 98% of diagnosis of prosthetic infection[24,27]. Finally it should be emphasized that the ESR has no diagnostic value in acute infections (< 6 wk) because it normally remains elevated after surgery for several weeks. Positive minor criteria are CRP > 100 mg/L in acute infections, and CRP > 10 mg/L and ESR > 30 mm in chronic infections[11,13].

DIAGNOSTIC ARTHROCENTESIS: SYNOVIAL FLUID ANALYSIS, LEUKOCYTE ESTERASE AND SYNOVIAL FLUID CULTURE

After the initial approach to the suspected diagnosis of prosthetic infection, including clinical history, physical examination, and initial laboratory and imaging studies, the next step is a diagnostic arthrocentesis, specifically a cell count to determine percentage of polymorphonuclear leukocytes (PMN), leukocyte esterase levels and a synovial fluid culture[13,21]. According Parvizi *et al*[13] a percentage of PMN above 65% has 97% sensitivity and 98% specificity for the diagnosis of prosthetic infections. As for the leukocyte count in the synovial fluid, figures above 4200/μL have sensitivity of 84% and a specificity of 93% for the diagnosis of infection.

The leukocyte esterase dipstick test is a fast, cheap and reproducible test. It consists of dipping a urinary test strip in the previously collected synovial fluid, leaving it submerged for two minutes and then interpreting the result according to color change. Leukocyte esterase is an enzyme released by neutrophils in response to infection[10,28]. It is reported that a leukocyte esterase value of ++ has a sensitivity of 81%, specificity of 100%, a positive predictive value of 100%, and a negative predictive value of 93% for the diagnosis of prosthetic infection.

The synovial fluid culture is a routine test within the studies in diagnostic arthrocentesis, performed to ensure specific antibiotic treatment for the infecting pathogen. This study has a sensibility of 86%-92% and a specificity of 82%-97%[29,30]. The use of a Petri dish is preferred because of its sensibility (90.92%) over intraoperative cultures in swabs or sterile containers (77%-82% sensibility)[31]. For optimal results, the following recommendations are made: (1) withhold antimicrobial therapy 2 wk prior sampling; and (2) prolong incubation period cultures at least two weeks for a definitive result[32,33]. However, it should be emphasized that the preoperative dose of antibiotic prophylaxis should not be suspended because it does not affect the sensitivity of intraoperative culture, in case the necessary diagnostic arthrocentesis sample was not obtained[34]. The full analysis of synovial fluid: Leukocyte count, PMN percentage, leukocyte esterase, and synovial culture, are part of the minor criteria for diagnosis of prosthetic infection and should be taken routinely in every patient[1,11,13].

HISTOPATHOLOGY

Another minor criterion for the diagnosis of periprosthetic infection is tissue biopsy[1,11,13]. As definition, a biopsy is considered positive when: It contains 5-10 PMN per high-power field in at least 5 different fields[21]. There are low-virulence bacteria that may be present in the simple and be reported as an inflammatory reaction or fibrosis. These bacteria are *Propionibacterium acnes* and coagulase-negative staphylococci, and may not be reported as positive findings. For this study, it is recommended to: (1) send 3-6 samples; and (2) take the sample with dissection techniques without the use of cautery (risk of false positives)[11].

TREATMENT

The management of prosthetic infection requires surgical intervention and prolonged periods of intravenous or oral antibiotics[1,35]. There is a lot of basic science and clinical research dedicated to the treatment of prosthetic infections; nonetheless, there are still many doubts as to how to treat them. Multidisciplinary management (orthopedist, infectious disease specialists, plastic surgeons) is of vital importance in these cases, as is following the consensus of therapeutic guidelines to diminish costs and morbidity and mortality in the patient[1]. There are several surgical options for treating prosthetic infections depending on the type of infection, virulence of the pathogen, and health status of the patient: (1) debridement, irrigation and cleaning with retention of the prosthesis; (2) single-stage revision surgery; (3) two-stage revision surgery; (4) arthrodesis; and (5) amputation[1]. So far, there are no randomized clinical trials where these surgical techniques are evaluated; most studies include patients from only one hospital, are non-comparative and decisions are based on cohort studies or case-control studies[1,36]. No matter the method of treatment, a prosthetic infection is not considered an emergency procedure (except in patient with sepsis). The patient must be in optimal condition for surgery, have normal glycaemia, hemoglobin > 10 mg/dL, and should be in optimal conditions for surgery[37].

TREATMENT: DEBRIDEMENT, IRRIGATION AND CLEANING WITH IMPLANT RETENTION

This technique has specific indications: (1) infection < 30 d in duration; (2) implants without evidence of loosening; (3) acute hematogenous infection; and (4) that the prosthesis was placed < 3 mo prior[1,38]. Contraindications include: (1) wound not closing on first intention; (2) presence of a fistula; and (3) evidence of prosthesis loosening. Relative contraindications are: (1) infection with highly virulent organisms (Methicillin-resistant *Staphylococcus aureus* MRSA); (2) polymicrobial infection; and (3) immunocompromised patients[17,39,40]. In a systematic review by Romanò *et al*[41] it was estimated that the success rate with this method varies between 0%-89%. There are factors that increase the success rate of the procedure such as infection by organisms of low virulence, rapid surgical treatment of patient with acute symptoms (less than 72 h and antibiotic treatment administered in the first month post-debridement[17,38,42,43].

During surgery, the same approach that was used for the placement of the prosthesis is performed[37,44]. By incising the deep dissection plane a better visualization of the structures is achieved[45]; the mobile components of the prosthesis are removed. When the modular components are removed, access to the surfaces underneath is achieved[17,38,46,47], 3-6 samples for culture and histology studies are taken[1,13], then the surgical site is irrigated with 6-9 L to avoid trauma to adjacent structures[37,48].

Medical treatment with antibiotic therapy is critical after surgery[17,43]. Various authors recommend rifampin combination with the antibiotic of choice. This is due to the action of rifampicin against biofilm, although there is no consensus as to when is the best time to start this treatment; several authors recommend initiating use in conjunction with intravenous antibiotic therapy in order to reduce the risk of selecting resistant mutants, others recommend to start rifampicin when oral antibiotics are started[49-51]. In a double-blind study by Zimmerli *et al*[51], acute infections by *Staphylococcus aureus* associated to orthopedic implants were treated with debridement, irrigation, cleaning and implant retention, combined with ciprofloxacin (750 mg/12 h) and rifampicin (450 mg/12 h) compared against ciprofloxacin as monotherapy (750 mg/12 h); finding a cure rate (100% hip and knee replacement 53%) higher when rifampicin is added with $P < 0.05$ at 35 mo follow-up[51]. When the microorganism is *Staphylococcus aureus* or coagulase-negative *Staphylococcus* and the germ is sensible, several studies recommend the combination of rifampin with fluoroquinolones[43,50-53]. Within fluoroquinolones, the one that best interacts with rifampicin is levofloxacin[54]. When talking about a MRSA, available information is very limited, however, studies report good results with the combination with rifampicin[55]. The combination of linezolid plus rifampicin reported cure rates of 60%[56-58]. However, its use is not recommended for more than six weeks due to toxicity and follow-up serum levels are necessary[59].

As for the duration of antibiotic therapy, the current trend is an initial intravenous therapy of 2-6 wk maximum, followed by 3 to 6 mo of oral antibiotics depending if it is a total hip or knee replacement[1,35]. The rapid change of intravenous to oral antibiotics (7-15 d) allows an early discharge for the patient and avoids catheter-associated infections[49]; this reports a success rate of over 70%[46,50,53]. Some authors recommend a treatment with intravenous antibiotics of less than 3 mo with similar success rates of over 70%[50,60]. However, it is an issue that is still under discussion and more information is needed to this[49].

TREATMENT: SINGLE-STAGE REVISION SURGERY

This type of procedure is not common in the United States, it is more common in Europe[1,61]. The indications for this technique are: (1) relatively healthy patients; (2) insignificant bone loss; (3) viable soft tissue; (4) low virulence microorganism (sensitive *Streptococcus aureus*, *Enterococci*, not infections by *Pseudomonas* or gram-negative bacteria); and (5) that the microorganism is susceptible to oral antibiotics with excellent bioavailability[1,61,62]. The advantages of this technique are: (1) lower cost for the patient/hospital/insurance system; (2) avoidance of a second surgery (in comparison with two-stage revision surgery); and (3) lower morbidity rates[63].

The technique consists of removing all of the prosthetic components including the cement (polymethyl methacrylate) aggressive debridement of soft and bone tissue (this being the most important factor). The placement of a new prosthesis, using antibiotic-loaded cement. This technique reports a success rate above 80%[63-65].

The medical treatment for single-stage revision surgery consists of administration of specific intravenous antibiotic treatment for 2-6 wk combined with oral rifampicin and changing the treatment to oral antibiotics for 3 mo. The success rate for this regimen is calculated between 80%-100% and two different approaches for treating these patients are described: (1) identification of the pathogen previous to surgery, followed by 4-6 wk of intravenous/oral antibiotic treatment (high bioavailability) followed by replacement of the prosthesis; and (2) in aseptic loosening, the prosthetic infection is confirmed by cultures, followed by intravenous antibiotic treatment combined with rifampicin[62,66,67].

TREATMENT: TWO-STAGE REVISION SURGERY

This is the technique of choice in the United States for the treatment of chronic periprosthetic infections[68-71]. The ideal patient and the indications for this technique

are: (1) chronic prosthetic infection; (2) insignificant bone loss; (3) patient in adequate conditions for surgery; (4) patient willing to undergo two surgeries; (5) patients with active fistula; and (6) high-virulence microorganisms (MRSA, Candida)[35,68,72]. This technique reports a success rate of 87%[1,73].

This surgical technique consists of aggressive debridement, removal of all prosthetic components including the cement (polymethyl methacrylate). Subsequently, a cement spacer with antibiotics is placed in block or articulated (to keep space and avoid future soft tissue contractures)[74,75]; in the second stage the cement spacer is removed and a new prosthesis is placed only if there is no evidence of infection. In case of infection, debridement, irrigation and cleaning should be performed again.

Regarding the medical treatment and the time of placement of the second prosthesis, reports vary from two to several months[70,74]. The most used strategy is 4-6 wk of intravenous antibiotic treatment (6 wk for *Staphylococcus aureus*) followed by 2-8 wk with no antibiotic treatment, obtaining good results[76-79]; in this case, rifampicin is not used, since the components with biofilm were removed[1].

TREATMENT: ARTHRODESIS

This is a useful treatment but has few indications; it involves the arthrodesis of the limb to allow ambulation and avoid amputation. The indications for this treatment are: (1) non-walking patients; (2) significant bone loss; (3) little and poor quality soft tissue; (4) high-virulence infections (low bioavailability antibiotics); (5) poor general condition of the patient; and (6) failure of two-stage revision surgery[1]. Arthrodesis is achieved by an intramedullary rod or an external fixator[80]. An eradication rate of 60%-100% is reported. Medical treatment involves the administration of intravenous or oral antibiotics (high bioavailability) for 4-6 wk[1].

TREATMENT: AMPUTATION

This treatment is reserved for select group of patients and its indications are: (1) necrotizing fasciitis (not responding to debridement); (2) severe bone loss; (3) soft tissue defect that could be closed primarily; (4) failed attempts at resection and arthrodesis; and (5) non-walking patients[1,73,81]. The technique consists of amputation or disarticulation above the affected areas. The medical treatment consists of antibiotic treatment for 24-48 h if clean and non-contaminated edges were achieved during surgery. In case of bacteremia, sepsis or inadequate debridement, intravenous or oral antibiotic treatment should be continued for 4-6 wk[1].

FUTURE MANAGEMENT OF PROSTHETIC INFECTIONS

Despite all initiatives and actions against prosthetic infections, the general incidence of infection ranges between 1%-2%[3,4]. Most actions are focused on improving the diagnostic tools and to combat biofilm[4,13].

Regarding the future of diagnostic imaging studies, the positron emission tomography (PET) scan is the imaging study that provides the most information for the diagnosis of prosthetic infections. The problem with PET scan is the variability of results that has been reported. In a meta-analysis by Kwee *et al*[82] composed of 11 studies, the PET scan reported a sensibility of 82.1% and a specificity of 86.6% for the diagnosis of prosthetic infection, concluding that there was great heterogeneity in the percentages reported by the studies. However, there are more recent studies that report a sensibility of 95% and a specificity of 98%[83]. More studies are needed to find the real value of PET scan for it to be a part of the diagnostic tools for prosthetic infections[13].

The most important biomarkers for the diagnosis of prosthetic infection are CRP and ESR[10,11,13]. However, interleucin-6 (IL-6) has been reported as an excellent marker for prosthetic infection, even above CRP and ESR. The advantage IL-6 offers is a return to normal levels within days, compared with weeks for CRP and months for ESR[84].

Diagnostic arthrocentesis is the method from which samples are taken for evaluating major and minor criteria for prosthetic infection[10,13]. Research shows that a CRP ELISA of synovial fluid is superior compared to serologic CRP, with a sensibility and specificity of 85%-97% (synovial CRP ELISA) *vs* 76%-93% (serologic CRP)[85]. But nevertheless, the best biomarker obtained from synovial fluid with reports of a sensibility and specificity of 100% is alpha-defensin[86,87]. This marker is a peptide secreted by the cells in response to microbial byproducts. The advantage it offers is that it is not influenced by inflammatory response nor by antibiotics; it is necessary to keep researching this test for it to be recommended generally[86,87].

As to perioperative tools/strategies to lower the periprosthetic infection there is the covering of prosthetic surfaces with silver ions. It has been reported that silver ions have antimicrobial properties when used in cream, gel and impregnated gauzes for the treatment of ulcers and wounds[88,89]. In a study by Gordon *et al*[90] the team designed a metallic prosthesis impregnated with silver polymers which showed *in vitro* activity against biofilm. Another strategy is the covering of the prosthesis with antibiofilm agents; biofilm is defined as a protective membrane of polysaccharides, polypeptides and nucleic acids that create an ideal microenvironment for the reproduction of bacteria and makes them resistant to antibiotics and the patient's immune system[91,92].

Extensive research has been made about therapies directed specifically to combating the physical integrity of the biofilm such as Deoxyribonuclease Ⅰ (DNase Ⅰ) and Dispersin B[93]. DNase Ⅰ degrades extranuclear DNA, which causes the firmness and stability of the biofilm. Dispersin B is directed against the intracellular adhesin produced by the biofilm[94]; its effects have been proved

against *S. aeureus, S. epidermidis,* and *E. coli*[94,95].

Regarding intraoperative therapies; disposable antibacterial coating (DAC) is used in the bone-prosthesis interface. DAC is an hydrogel made of hyaluronic acid and polylactic acid to which specific antibiotics against the microorganism can be added; a great advantage since a high dose of antibiotics are added to the surgical site. This gel is smeared on the prosthesis (with no cement) prior to placement and it is reported to release antibiotics for up until 96 h[96].

CONCLUSION

Prosthetic infections continue to be a devastating complication for patients, health systems and the medical teams who handle these cases. Despite the progress made in diagnostic tools and the unification of criteria for creating treatment algorithms, the management of these cases is still a challenge for the orthopedic surgeon. It is expected that in the near future, better diagnostic tools for prosthetic infections will be created.

Clinical suspicion of the orthopedic surgeon is the cornerstone for achieving a quick diagnosis and choosing the ideal treatment; early diagnosis in acute infections is essential to preserve the prosthesis. In chronic infections, two-stage revision surgery is the treatment of choice in the vast majority of cases.

The current tendency is to reduce the intravenous antibiotic treatment when the bacteria involved are susceptible to oral antibiotics with ample bioavailability and to asses the duration of antibiotic treatment according to the patient's clinical response, with satisfactory results, with the benefit of shorter hospital stays, decreased complications of catheter use and reduced side effects of prolonged intravenous antibiotic therapy.

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ORIGINAL ARTICLE

Basic Study

Antioxidant enzyme profile of two clinical isolates of *Entamoeba histolytica* varying in sensitivity to antiamoebic drugs

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Author contributions: Iyer LR and Paul J conceived and designed the experiments; Iyer LR, Banyal N and Naik S performed the experiments; Paul J contributed reagents, material, analysis tools; Iyer LR, Banyal N and Paul J analyzed the data; Iyer LR and Paul J prepared the manuscript; Iyer LR and Paul J participated in revising the manuscript.

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Abstract

AIM

To study the sensitivity and antioxidant enzyme response in two clinical isolates of *Entamoeba histolytica* (*E. histolytica*) during treatment with antiamoebic drugs, auranofin and metronidazole.

METHODS

E. histolytica were isolated from stool samples and maintained in Robinson's biphasic culture medium. Clinial isolates were maintained in xenic culture medium, and harvested for determination of minimum inhibitory concentrations to the two antiamoebic drugs, Metronidazole and Auranofin using microtiter plate tests. The percent survival of the two isolates were determined using the trypan blue cell count. Isolate 980 was treated with 70 μmol/L and 2 μmol/L while isolate 989 was treated with 20 μmol/L and 0.5 μmol/L of metronidazole and auranofin respectively for 24 h. Fifty thousand cells of each isolate were harvested after 24 h of treatment for analysis of the mRNA expressions of the antioxidant enzymes, thioredoxin reductase, peroxiredoxin and FeSOD using the specific primers. Cell lysate was used for determination of enzyme activity of thioredoxin reductase by measuring DTNB reduction spectrophotometrically at

412 nm.

RESULTS

Minimum inhibitory concentration of the clinical isolates 980 and 989 for auranofin was 3 μmol/L and 1 μmol/L respectively while that for metronidazole was 80 μmol/L and 30 μmol/L respectively. Thioredoxin reductase, peroxiredoxin and FeSOD expression levels were significantly reduced in the isolate 980 when treated with Auranofin. Metronidazole treatment showed a down regulation of thioredoxin reductase. Though not significant both at the mRNA and the enzyme activity levels. Peroxiredoxin and FeSOD however remained unchanged. Auranofin treatment of isolate 989, showed an upregulation in expression of thioredoxin reductase while Peroxiredoxin and FeSOD did not show any change in expression. Upon treatment with metronidazole, isolate 989 showed an increase in thioredoxin reductase expression. Peroxiredoxin and FeSOD expressions however remain unchanged both at mRNA and enzyme activity level.

CONCLUSION

Clinical isolates from New Delhi NCR region show different sensitivities to antiamoebic drugs. Auranofin is effective against isolate showing higher tolerance to metronidazole as shown by its inhibition in thioredoxin reductase activity.

Key words: Metronidazole; *Entamoeba histolytica*; Amoebiasis; Thioredoxin reductase; Minimum inhibitory concentrations; Auranofin

Core tip: Due to overuse of the mainstay drug against amoebiasis in an endemic country like India, there are concerns regarding the development of resistance towards metronidazole by the parasite. When *Entamoeba histolytica* (*E. histolytica*) from stool samples of diarrheal patients were cultivated in xenic medium, two clinical isolates of *E. histolytica* showed differential tolerance to the commonly used drug metronidazole. A new drug Auranofin was found to be effective on the isolate with higher tolerance to metronidazole. This was shown by inhibition of the antioxidant enzyme thioredoxin reductase as monitored by mRNA expression of *TrxR* gene and its enzyme activity.

Iyer LR, Banyal N, Naik S, Paul J. Antioxidant enzyme profile of two clinical isolates of *Entamoeba histolytica* varying in sensitivity to antiamoebic drugs. *World J Clin Infect Dis* 2017; 7(2): 21-31 Available from: URL: http://www.wjgnet.com/2220-3176/full/v7/i2/21.htm DOI: http://dx.doi.org/10.5495/wjcid.v7.i2.21

INTRODUCTION

Amoebiasis is a disease caused by *Entamoeba histolytica* (*E. histolytica*), a protozoan parasite. It has been classified as category B priority biodefence pathogen by the National Institutes of health. This pathogen is effective even at a low infectious dose, and it has a high potential for transmission through contaminated food and water[1]. The parasite invades and destroys human tissue. It survives and proliferates in the human gut in an atmosphere of reduced oxygen. When the microaerophilic *E. histolytica* invades tissue, it is exposed to reactive oxygen species. It overcomes oxygen stress using antioxidant enzymes. *E. histolytica* lacks glutathione reductase enzyme, therefore a thioredoxin-linked system plays the major role to counter oxidative stress. This system is made up of proteins like peroxiredoxin, rubrerythrin, Iron containing superoxide dismutase, NADPH: Flavin oxidoreductase, and amino acids like L-cysteine, S-methyl-L-cysteine, and thioprolines[2].

The thioredoxin reductase/thioredoxin system (TrxR/Trx) protects sensitive proteins like serine acetyltransferase-1 against oxidative stress *in E. histolytica.* Peroxiredoxin, a central redox regulatory and antioxidant protein in Eh is the terminal peroxidase reducing H_2O_2 and depends on electrons provided by the TrxR/Trx system[3]. The Eh TrxR is versatile, and can use NADPH or NADP as its reducing cofactor and there is evidence that it protects the parasite from reactive oxygen (ROS) and reactive nitrogen species. It is therefore an ideal drug target[4].

Metronidazole is a 5-nitroimidazole derivative and is used to treat infections by anaerobic bacteria and protozoans like amoeba and giardia. This drug shows selective toxicity to anaerobic organisms as they possess metabolic pathways of low redox potential. Metronidazole is converted to its active form when its nitro group is reduced to an anion radical in Entamoeba cell. The active form of the drug is highly reactive and known to form additives with proteins and DNA causing loss of their functions[5]. Other enzymes have also been reported to be metronidazole activating nitroreductases, out of which thioredoxin reductase is one such enzyme. Jeelani *et al*[6] identified two additional NADPH dependent nitroreductases having metronidazole reducing activity. The reduced form of active metronidazole is detoxified inside the cell by the action of Superoxide dismutase, forming hydrogen peroxide and oxygen. Peroxiredoxin scavenges the hydrogen peroxide converting it to water. *E. histolytica* with induced resistance to metronidazole have been reported in literature earlier. Induced resistance to metronidazole leads to increased activity of FeSOD and peroxiredoxin and reduced expression of ferredoxin and Flavin reductase[7,8].

Auranofin is an oral gold salt and was first used to treat rheumatoid arthritis. The anti-parasitic activity of auranifin is due to the monovalent gold molecules which inhibit thioredoxin reductase. Auranofin was found to be active at nanomolar concentrations against various parasites, including *E. histolytica*, Giardia, *Trypanosoma brucei*, *Leishmania infantum*, *L. major*, and *P. falciparum*[4]. Auranofin was also found to be active against *G.lamblia*

isolates pathogenic to humans in the 4-6 μmol/L range and against metronidazole resistant strains of giardia. Auranofin reportedly blocked the activity of giardial thioredoxin oxidoreductase. It was found effective *in vivo* in eradicating infections in different rodent models[9].

Auranofin has been recently been identified by a High Throughput Screening technique as active against trophozoites of *E. histolytica* and cysts of *E. invadens.* Auranofin inhibits *E. histolytica* thioredoxin reductase and it was shown that thioredoxin reductase protects trophozoites from oxidative attack and therefore in auranofin treated cells, thioredoxin was found in the oxidized state[10,11]. Auranofin has received orphan drug status and clinical trials are being carried out to treat amoeba and giardia infections. It shows promise as a broad-spectrum drug against *Entamoeba, giardia* and *cryptosporidium*, which are a major cause of diarrhea.

Both metronidazole and auranofin are combated by the thioredoxin reductase based enzyme system in Entamoeba. In this work, we therefore studied the sensitivity to auranofin of two isolates of *Entamoeba* from New Delhi, isolate 989 which is sensitive to metronidazole and isolate 980, which showed tolerance to metronidazole. We compared the activity and mRNA expression levels of thioredoxin reductase and the mRNA expression levels of peroxiredoxin and FeSOD in the above isolates, upon treatment with two antiamoebic drugs.

MATERIALS AND METHODS

Isolation and maintenance of clinical isolates of E. histolytica in xenic culture medium

The clinical isolates were obtained from patient samples from Safdarjung Hospital, New Delhi, NCR region. They were isolated from stool specimens and cultured in Robinson's biphasic culture medium with Escherichia coli[11,12]. They were subcultured every 48 h.

Isolation of DNA from xenic culture and identification of E. histolytica

The *E. histolytica* isolates were grown in xenic culture and cells were harvested for DNA isolation and pelleted at 600 g at 4 ℃. The pellets were stored in 70% ethanol at -20 ℃ till DNA isolation. QIA Amp DNA minikit was used for extracting genomic DNA (Qiagen catalog No. 51366). PCR amplification was used for strain identification from the genomic DNA using strain specific primers described by Srivastava *et al*[13]. The strains identified were either *E. histolytica* or *E. dispar.*

Microtitre plate tests to determine the minimum inhibitory concentration of isolates

Microtitre plate tests to determine the minimum inhibitory concentration (MIC) of Auranofin to Indian isolate of *E. histolytica* were performed using a method modified from the one described by Upcroft *et al*[14]. Different drug dilutions were created in the liquid phase of the xenic Robinson's medium in wells of microtiter plates and a fixed number of cells of the isolate was inoculated in the wells. A low oxygen environment was created using a sachet and bag system and cell growth was monitored in the wells under the microscope, without aerobic exposure at different time intervals. A score was given using a prefixed scoring system. For example, in case of isolate 980, in 24 h the control wells were fully confluent with live motile cells, so a score of ++++ indicated that.

The scores were as follows: (1) ++++ = (confluent well, covered with live motile cells); (2) +++ = 50%-70% (almost confluent well filled with live motile cells); (3) ++ = (30%-50% well coverage, few cells motile); (4) (+) ⩽ 20% well coverage with rounded cells; and (5) (-) = dead and disintegrated cells. The MIC was the lowest concentration giving a + score, after 48 h.

Percentage survival of clinical isolates 980 and 989 after antiamoebic drug treatment using trypan blue cell count

To determine the percentage survival of the clinical isolates, each isolate was expanded in around 8 culture tube and harvested after 48 h to get a harvest of about approximately five lakh cells. Fifty thousand cells each were inoculated into twelve culture tubes, 6 tubes had the required drug concentration, while 6 tubes remained untreated to be used as controls.

The tubes were incubated at 35.5 ℃ for 15 h, 24 h and 48 h after inoculation. After each time period, the cells were harvested from two drug treated and two untreated controls and pelleted separately. Each pellet was dissolved in 1 mL medium and cell counting was done using a hemocytometer. Dilutions were made in PBS to obtain optimum count of up to 20 cells per quadrant in the hemocytometer. A 0.5 percent trypan blue solution was added in the ratio of 1:1 to the diluted cells and incubated for 1 min, before loading it to the hemocytometer and counting. Blue stained dead cells were not counted. Duplicate counts for each time period, in the treated cells and untreated controls were calculated. The mean and standard deviation values of the treated cells were plotted as percent of the untreated control and expressed as percent survival.

Short term treatment of clinical isolates 980 and 989 with antiamoebic drugs, auranofin and metronidazole

To give a short term treatment with the antiamoebic drugs, eight tubes each of the isolate 980 and isolate 989 were cultured in Robinson's medium for 24 h. The cells were pelleted at 600 g for 5 min at 4 ℃. The cells were counted and approximately 50000 cells each were suspended in the liquid phase of sixteen fresh tubes each per drug per isolate containing Robinsons medium, having the antiamoebic drugs in the required concentrations. This was 70 μmol/L and 20 μmol/L of metronidazole and 2 μmol/L and 0.5 μmol/L of Auranofin for isolate 980 and isolate 989 respectively. For each group of 16 tubes the complete medium was dispensed and they were incubated at 35.5 ℃ for 24 h. Subsequently cells were pelleted in the same way as above in RNAase free 50 mL

Table 1 Primers used in RT-PCR of *Entamoeba histolytica* thioredoxin reductase, peroxiredoxin, FeSOD and 18SrRNA

S.No.	Primer	Sequence	Tm	Amplicon size	Ref.
1	Thioredoxin reductase (TrxR)	F-5′GTAATATTCATGATGTTGT3′	48 ℃	204 bp	[4]
	Accession no (EHI_155440)	R-5′CATCATTAATTCATTTTCCA3′	48 ℃		
2	*Eh* Peroxiredoxin (Prx)	F 5′AAATCAATTGTGAAGTTATTGG3′	53.6 ℃	100 bp	[16]
		R 5′TCCTACTCCTCCTTTACTTTTA3′	56.8 ℃		
3	FeSOD	F 5′ACAATTACCTTATGCTTATAA3′	52 ℃	240 bp	[16]
	Accession number (XM_643735.2)	F 5′TCCACATCCACACATACAAT3′	54 ℃		
4	*Entamoeba histolytica* 18s ribosomal RNA gene	F 5′TCAGCCTTGTGACCATACTC3′	61.7 ℃	200 bp	[16]
		F 5′AAGACGATCAGATACCGTCG3′	68.9 ℃		

conical centrifuge tubes. After decanting the supernatant, the cells pellets were stored at -80 ℃ in Trizol reagent for RNA isolation. Negative control RNAs were prepared for all the treated groups, which consisted of sixteen tubes of Robinson's medium, without the inoculum (blank), harvested after incubation for 24 h at 35.5 ℃ and RNA was extracted following the same procedure as that for the test vials.

Expression of antioxidant enzymes thioredoxin reductase, peroxiredoxin and FeSOD by semi quantitative RT-PCR

Primers used for RT-PCR: The primers sequences for the RT-PCR amplification of thioredoxin reductase, peroxiredoxin and FeSOD and 18S rRNA are listed in Table 1.

Isolation of mRNA for semiquantitative RT-PCR: The total RNA was isolated from the untreated and treated harvested cells using Trizol reagent (Invitrogen) and treated with DNase (Roche), following the manufacturers protocol. Total RNA from uninoculated culture medium, incubated at 35.5 ℃ for 24 h served as blank for RT-PCR and gel electrophoresis.

Semiquantitative RT-PCR: The total RNA isolated from the drug treated and untreated *E. histolytica* cells were used for RT-PCR. Promega random hexamer and MMLV RT enzyme was used for the reverse transcriptase reaction. 18S rRNA was used for normalization. RT-PCR amplification of peroxiredoxin and 18S rRNA was carried out together as annealing temperatures were similar and their product sizes varied by 100 base pairs and could be easily separated in gel chromatography while RT-PCR of thioredoxin reductase and of FeSOD were carried out separately. The PCR reactions had an initial denaturation at 94 ℃ for 5 min, each targeted gene was subjected to 30 cycles of amplification followed by 1 min annealing. The annealing temperature was 50 ℃ for 18SrRNA as well as peroxiredoxin and 48 ℃ for FeSOD as well as TrxR. Extension temperature was 72 ℃ for 1 min and final extension was for 5 min at the same temperature.

A 1.2% agarose gel was used to run the amplified products and stained with ethidium bromide and quantified using the Alpha Imager gel documentation. Three repeats of the experiment for each of untreated and treated isolate was performed.

Spot densitometry: Alpha Ease FC software was used to quantify the bands obtained during electrophoresis for the amplified mRNAs. The densitometric values of the bands obtained for thioredoxin reductase, Peroxiredoxin and FeSOD were expressed as percents of the 18S rRNA band density.

Statistical analysis: The mean, standard error and paired *t*-test of treated as well as untreated groups were determined for the metronidazole and auranofin treated isolates.

Determination of thioredoxin reductase activity in cell extracts of E. histolytica clinical isolates

Preparation of cell extracts: Short term metronidazole and Auranofin stress was given to the clinical isolate 980 and 989, and the cells were harvested after 24 h, as described above. The pellet was washed in PBS, supernatant was discarded, pellet volume measured and pellet transferred to a Dounce homogenizer after suspending it in Tris buffer [100 mmol/L Tris/HCl (pH 7.5)]. A protease inhibitor cocktail in the ratio 1:100 was added at this step to prevent breakdown of proteins. The homogenizer was placed in ice. The cells were then disrupted with 25 strokes of the pestle of the dounce homogenizer. The lysates were then transferred to an Eppendorf tube and pelleted at 20000 g for 10 min at 4 ℃. The supernatant was stored at -80 ℃ until the protein and thioredoxin reductase assay was performed.

Determination of Thioredoxin reductase activity: Thioredoxin reductase activity was determined in the cell extracts by the spectrophotometric measurement of DTNB reduction at 412 λ by the action of *E. histolytica* thioredoxin reductase. The assay was carried out in microtitre plates. The assay mixture consisted of 100 mmol/L potassium phosphate (pH 7.0), 10 mmol/L EDTA and 0.24 mmol/L NADPH and 3 mmol/L DTNB. Measurements were made under aerobic conditions at 25 ℃ in a Thermoscientific Multiscango plate reader, using an enzymatic kinetic program. Ten readings per sample were taken at an interval of 30 s each. The data was expressed as units of enzymes per mg protein, where each unit causes an increase in λ 412. Each assay was performed thrice, and the data was expressed as

Table 2 Representative minimum inhibitory concentration plate tests of clinical isolates of *Entamoeba histolytica* to antiamoebic drugs

Concentration	15 h			24 h			48 h		
	W-1	W-2	W-3	W-1	W-2	W-3	W-1	W-2	W-3
MIC of *Entamoeba histolytica* clinical isolate 980 to auranofin = 3 μmol/L									
Control	+++	++++	+++	+++	+++	+++	+++	++	++
DMSO control	+++	+++	++++	+++	+++	+++	+++	++	++
1 μmol/L	++	+++	++	++	++	++	++	++	++
2 μmol/L	+	++	++	++	++	++	++	+	+
3 μmol/L	+	+	+	+	+	-	+	-	+
4 μmol/L	+	+	+	-	-	-	-	-	-
MIC of of *Entamoeba histolytica* clinical isolate 980 to metronidazole = 80 μmol/L									
Control	+++	++++	+++	+++	+++	+++	+++	++	++
DMSO control	+++	+++	++++	+++	+++	+++	+++	++	++
50 μmol/L	++	+++	+++	++	++	++	++	++	++
60 μmol/L	++	++	++	++	++	++	++	++	+
70 μmol/L	+	++	++	++	++	++	++	+	+
80 μmol/L	++	+	++	++	+	+	+	+	-
90 μmol/L	+	+	+	+	-	-	-	-	-
MIC of *Entamoeba histolytica* clinical isolate 989 to auranofin = 1 μmol/L (MIC determined to be 2 μmol/L)									
Control	+++	++++	+++	+++	+++	+++	+++	++	++
DMSO control	+++	+++	+++	+++	+++	+++	+++	++	++
1 μmol/L	++	+++	+++	++	+	+	+	+	+
2 μmol/L	++	++	+	+	+	+	+	-	-
3 μmol/L	+	+	+	-	-	-	-	-	-
4 μmol/L	-	-	-	-	-	-	-	-	-
MIC of of *Entamoeba histolytica* clinical isolate 989 to metronidazole = 30 μmol/L									
Control	+++	++++	+++	+++	+++	+++	+++	++	++
DMSO control	+++	+++	+++	+++	+++	+++	+++	++	++
10 μmol/L	++	+++	+++	++	++	++	++	++	++
20 μmol/L	++	+++	++	+	++	++	++	++	+
30 μmol/L	++	++	+	+	+	+	+	+	-
40 μmol/L	+	+	+	-	-	-	-	-	-

The lowest concentration giving a + score, after 48 h, was the minimum inhibitory concentration. W-1, W-2, and W-3, represent the triplicate wells. Scores are ++++: Confluent well, covered with live motile cells; +++: 50%-70% almost confluent well filled with live motile cells; ++: 30%-50% well coverage, few cells motile); +: ≤ 20% well coverage with rounded cells; -: Dead and disintergrated cells.

mean ± SD for three independent experiments.

Protein estimation was carried out in the cell extracts using the colorimetric Bichinchonic acid assay in 96 well microtiter plates, where the absorbance was measured at 562 nm. The linear range of this assay was 200-100 μg/mL.

Statistical analysis: Mean ± SE of of all the data was determined and compared between untreated *E. histolytica* and cells treated with auranofin and metronidazole. A paired student's *t* test was used for comparison between the groups.

Ethical consideration: Informed consent for obtaining stool samples and ethical permission was taken from participants for the study.

RESULTS

Determination of MICs of Antiamoebic drugs to clinical isolates

MIC of isolate 980 to Auranofin and Metronidazole: Table 2 shows a representative plate test to determine the MIC of isolate 980 to Auranofin. MIC of Auranofin for isolate 980 was 3 μmol/L, the lowest concentration where live cells were present at 48 h. This experiment was carried out five times in triplicate wells and in all attempts the MIC was found to be 3 μmol/L. Table 2 shows a representative plate test to determine the MIC of isolate 980 to metronidazole. MIC was 80 μmol/L, the lowest concentration where live cells were present at 48 h. This experiment was carried out ten times in triplicate wells. The MIC was found to be 80 μmol/L in most attempts, while in a couple of attempts the MIC was 100 μmol/L.

MIC of isolate 989 to auranofin and metronidazole: Table 2 shows a representative plate test to determine the MIC of isolate 989 to Auranofin. MIC of Auranofin in isolate 989 was found to be 1 μmol/L, the lowest concentration where live cells were present at 48 h. Table 2 shows a representative plate test to determine the MIC of metronidazole in isolate 989. The MIC was found to be 30 μmol/L, the lowest concentration with live cells at 48 h. This experiment was carried out in triplicate wells and in 5 attempts, and each time the same MIC was observed.

MIC of metronidazole and auranofin in other

Table 3 Minimum inhibitory concentrations for clinical isolates of *Entamoeba histolytica* to metronidazole and auranofin in drug susceptibity assays

Isolate	MIC metronidazole	Range (μmol/L)	MIC auranofin	Range (μmol/L)	No. of attempts
654	50 μmol/L	50-60	2 μmol/L	2-3	3
812	40 μmol/L	30-40	2 μmol/L	2-3	3
980	80 μmol/L	80-100	3 μmol/L	80-100	5 (auranofin) 10 (metronidazole)
989	30 μmol/L	30-40	1 μmol/L	1-2	5
5132	50 μmol/L	50-60	2 μmol/L	2-3	3
MS-96:3382	24 μmol/L	20-30	5 μmol/L	4-5	4

Table 4 Percent viability of clinical isolate 980 and 989 after treatment with metronidazole and auranofin

	15 h	24 h	48 h
Percent viability of clinical isolate 980 after treatment with metronidazole			
50 μmol/L metronidazole	61.8 ± 0.13	74.12 ± 14.1	70.23 ± 3.66
70 μmol/L metronidazole	53.06 ± 14.1	69.38 ± 3.13	60.68 ± 6.74
90 μmol/L metronidazole	47.31 ± 6.2	26.39 ± 10.7	24.3 ± 14.75
Percent viability of clinical isolate 980 after treatment with auranofin			
1 μmol/L auranofin	91.5 ± 0.26	25.17 ± 5.85	12.88 ± 1.63
2 μmol/L auranofin	56.6 ± 5.81	27.92 ± 5.84	0
3 μmol/L auranofin	47.74 ± 7.67	22.41 ± 4.62	0
Percent viability of clinical isolate 989 after treatment with metronidazole			
20 μmol/L	-	92.51 ± 2.79	65.22 ± 18.5
30 μmol/L	-	76.11 ± 17.13	25.39 ± 5.33
40 μmol/L	-	54.81 ± 0.57	0
Percent viability of clinical isolate 989 after treatment with auranofin			
0.5 μmol/L	-	45.47 ± 0.26	43.01 ± 2.33
1 μmol/L	-	36.63 ± 3.00	19.4 ± 2.95
2 μmol/L	-	29.16 ± 2.95	0

Values are expressed as percent of controls.

clinical isolates: Using the same microtiter plate test method described for 980 and 989, we performed a pilot study on other clinical isolates of *E. histolytica,* cultured in our laboratory to assess their MICs (Table 3). MIC's for metronidazole ranged from 30 μmol/L to 50 μmol/L, amongst these, isolate 980 showed a high tolerance to metronidazole with an MIC of 80 μmol/L and isolate 989 was more sensitive compared to the rest of the clinical isolates. MIC's for auranofin ranged from 1-3 μmol/L in the five clinical isolates studied. From the clinical samples maintained in xenic culture, two isolates, 989 and 980, representing a sensitive and tolerant population respectively to antiamoebic drug, metronidazole were selected for expression studies. These isolates were further tested for expression analysis using auranofin drug also.

Percent survival of clinical isolates 980 and 989 on treatment with antiamoebic drugs, metronidazole and auranofin using trypan blue cell count

The percent survival of the clinical isolate 980 after treatment with metronidazole, for different time periods is shown in Table 4. The survival of the treated isolate was expressed as percent of the untreated controls. A concentration where the effect of metronidazole was seen and yet enough viable cells could be harvested was 70 μmol/L and this concentration was used for further experiments on metronidazole stress. The percent survival of clinical isolate 980 with different concentrations of auranofin is shown in Table 4. The viability was expressed as percent of the untreated controls. At a concentration of 2 μmol/L of Auranofin there were 27% viable cells at 24 h. This concentration was chosen for further experiments. Percent survival of clinical isolate 989 on treatment with different concentrations of metronidazole is shown in Table 4. The survival of the treated isolate was compared with untreated control. It was observed that at 20 μmol/L metronidazole, 65% of cells survived in 48 h. This concentration was chosen for further experiments. Percent survival of isolate 989 during auranofin treatment, is shown in Table 4. Zero point five μmol/L auranofin treatment gave 45% survival in 24 h and 43% in 48 h. Treatment with 1 μmol/L auranofin reduced cell survival to 19% in 48 h in this isolate. Therefore 0.5 μmol/L treatment was given to the cells for further experiments in this isolate in order to harvest sufficient number of cells.

Expression of thioredoxin reductase in clinical isolates 980

Figure 1A shows the mRNA expression percent using semiquantitative RT-PCR of thioredoxin reductase in clinical isolate 980 after treatment with metronidazole and Auranofin. The paired columns compare the untreated and the treated isolate. The columns represent the

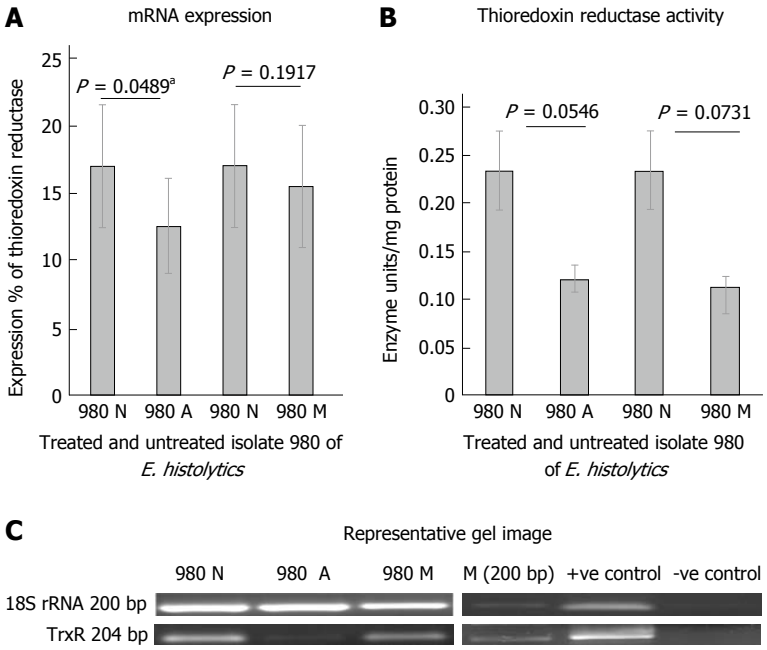

Figure 1 mRNA expression and activity levels of thioredoxin reductase in clinical isolate 980 of *Entamoeba histolytica* during treatment with antiamoebic drugs. A: Graphical representation of densitometric data from semiquantitative RT-PCR analysis of Thioredoxin reductase in clinical isolate 980. Untreated (980 N), auranofin treated (980 A) and metronidazole treated (980 M). Densitometric values are expressed as percent after normalizing with 18S rRNA. Each pair of columns show the data for untreated and treated isolate. Data are mean ± SE of three independent experiments. [a]$P < 0.05$ in case of auranofin treatment; B: Activity of thioredoxin reductase in clinical isolate 980, untreated (980 N), auranofin treated (980 A) and metronidazole treated (980 M). Each pair of columns show the enzyme activity in units/mg protein, for the treated and untreated isolate; C: Representative gel image: 980 N: Untreated isolate; 980 A: Auranofin treated; 980 M: Metronidazole treated. M: Marker; +ve C: HM-1: IMSS cDNA used as +ve control; -ve C: cDNA from blank medim used as -ve control; TrxR: Thioredoxin reductase; *E. histolytics: Entamoeba histolytica*.

spot densitometry data of thioredoxin reductase mRNA expression graphically. It is expressed as a percent of 18SrRNA, an internal control. TrxR expression in isolate 980 showed a downregulation on treatment with anti amoebic drugs, auranofin and metronidazole. This was statistically significant in case of auranofin treatment ($P < 0.05$). Figure 1B is a representative gel picture of the thioredoxin reductase mRNA expression in the treated and untreated isolate. Figure 1C shows the thioredoxin reductase enzyme activity in units/mg protein. The activity shows a decreasing trend on treatment with antiamoebic drugs in comparison to control, this decrease was however not statistically significant.

Expression of thioredoxin reductase in clinical isolate 989

Figure 2A shows the mRNA expression percent of thioredoxin reductase in clinical isolate 989 after treatment with metronidazole and auranofin, using semi quantitative RT-PCR. At mRNA level, an increase in expression (not significant) was observed when cells were treated with 0.5 μmol/L of auranofin. A similar increase in mRNA level was seen when treated with 20 μmol/L of metronidazole. Figure 2B shows a representative gel picture of the TrxR in treated and untreated isolate at mRNA level. 18S rRNA was used as an internal control. Figure 2C shows the Thioredoxin reductase activity on treatment of 989 with auranofin and metronidazole for 24 h. We observed a significant increase ($P = 0.036$) in TrxR activity when cells were treated with 0.5 μmol/L of auranofin. Similar increase was seen in case of metronidazole treatment however it was not significant.

Expression of peroxiredoxin and iron containing superoxide dismutase (FeSoD) in clinical isolate 980

The mRNA expression of peroxiredoxin in the isolate when treated with Auranofin and Metronidazole is shown in Figure 3A. It was observed that peroxiredoxin expression in isolate 980 was significantly down regulated at the mRNA level after treatment with auranofin. This decrease was significant when the data was analyzed using paired *t*-test ($P = 0.0228$). However, in case of metronidazole treatment no significant change was observed. Figure 3B shows a representative gel picture of the peroxiredoxin expression. Figure 3C shows the mRNA expression of FeSOD in isolate 980 after treatment with auranofin and metronidazole. There was a decreased FeSOD expression on treatment with auranofin. The decrease was significant with a *P* value of 0.0113. However, no significant change was observed when treated with metronidazole. Figure 3D shows a representative gel picture of FeSOD expression in 980. Internal control in both the experiments was 18S rRNA.

Expression of peroxiredoxin and FeSoD in clinical isolate 989

No significant change could be seen in the mRNA

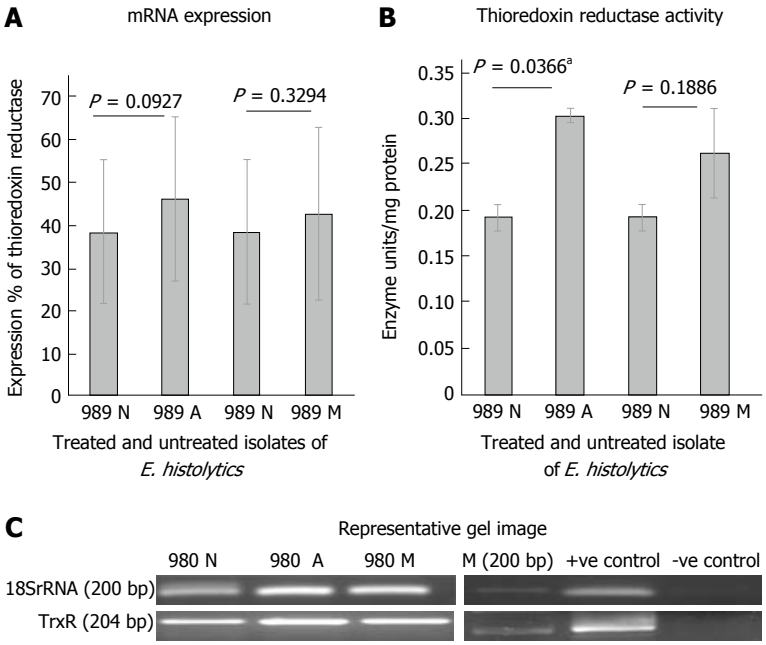

Figure 2 mRNA expression and activity levels of thioredoxin reductase in clinical isolates 989 of *Entamoeba histolytica* during treatment with antiamoebic drugs. A: Graphical representation of densitometric data from semiquantitative RT-PCR analysis of Thioredoxin reductase in clinical isolate 989, untreated (989 N), auranofin treated (989 A) and metronidazole treated (980 M). Densitometric values are expressed as percent after normalizing with 18S rRNA. Each pair of columns show the data for untreated and treated isolate. Data are mean ± SE for three independent experiments; B: Activity of thioredoxin reductase in clinical isolate 989, untreated (989 N) and after treatment with auranofin (989 A) and metronidazole (989 M). Each pair of columns show the data for untreated and treated isolate. Data are mean ± SE for three independent experiments. $^{a}P < 0.05$ in case of auranofin treatment; C: Representative gel image of thioredoxin reductase expression. 989 N: Untreated isolate; 989 A: Auranofin treated; 989 M: Metronidazole treated; M: Marker; +ve C: HM-1: IMSS cDNA used as +ve control; -ve C: cDNA from blank medim used as -ve control; TrxR: Thioredoxin reductase; *E. histolytics: Entamoeba histolytica*.

expression of either peroxiredoxin or FeSOD when the isolate 989 was treated with both antiamebic drugs, auranofin and metronidazole. Data not shown.

DISCUSSION

MICs of clinical isolates maintained in xenic cultures from New Delhi, NCR region, using this method ranged from 30-50 μmol/L for metronidazole, and 1-3 μmol/L for auranofin. In case of the axenic strains HM-1: IMSS the reported MICs using this method ranged from 12.5-25 μmol/L[14]. We report here for the first time on the MIC of auranofin in clinical isolates.

The clinical isolates showing different sensitivities to auranofin and metronidazole were further tested for their antioxidant activities upon treatment. It was observed in isolate 980, at a concentration of 2 μmol/L auranofin, the thioredoxin reductase mRNA expression was down regulated. This was further confirmed by a significant decrease in thioredoxin reductase enzyme activity at the protein level. Auranofin has been shown to inhibit *E. histolytica* HM-1: IMSS thioredoxin reductase[11]. Treatment of isolate 980 with 2 μmol/L auranofin also downregulated its peroxiredoxin expression and superoxide dismutase expression at the mRNA level.

It is known that the reaction catalyzed by superoxide dismutase converts reactive oxygen to H_2O_2 and the peroxiredoxin further detoxifies it to H_2O with the help of electrons provided by TrxR/Trx system[2]. TrxR enzyme is required for the reduction of thioredoxin which donates electrons to oxidized peroxiredoxin. It is likely that inhibition of TrxR leads to a general inhibition of the normal detoxification process in the parasite involving peroxiredoxin and superoxide dismutase. Debnath et.al have earlier shown by *in vitro* assays that auranofin treated *E. histolytica* cells were more susceptible to oxidative stress and accumulated more ROS[11]. Our data on clinical isolate 980 also showed that auranofin treatment significantly reduced expression of these antioxidant enzymes at the mRNA level and the enzyme activity of thioredoxin reductase at protein level was also reduced compared to untreated controls.

Treatment of isolate 980 with 70 μmol/L metronidazole, also reduced thioredoxin reductase expression at the mRNA level and also at the enzyme activity level though not significantly. Thioredoxin reductase enzyme plays a role in the activation of metronidazole by its nitro reductase activity[15]. This decrease in its expression may contribute to the higher metronidazole tolerance of isolate 980 compared to the other clinical isolate 989. The peroxiredoxin and superoxide dismutase expression of the isolate 980 at mRNA level did not show any significant change in expression after treatment with metronidazole for 24 h. This suggests that there is no increase in detoxification inside the cell. This further suggests that all metronidazole is not being converted to its active form, though the cells were exposed to a high metronidazole concentration due to downregulation

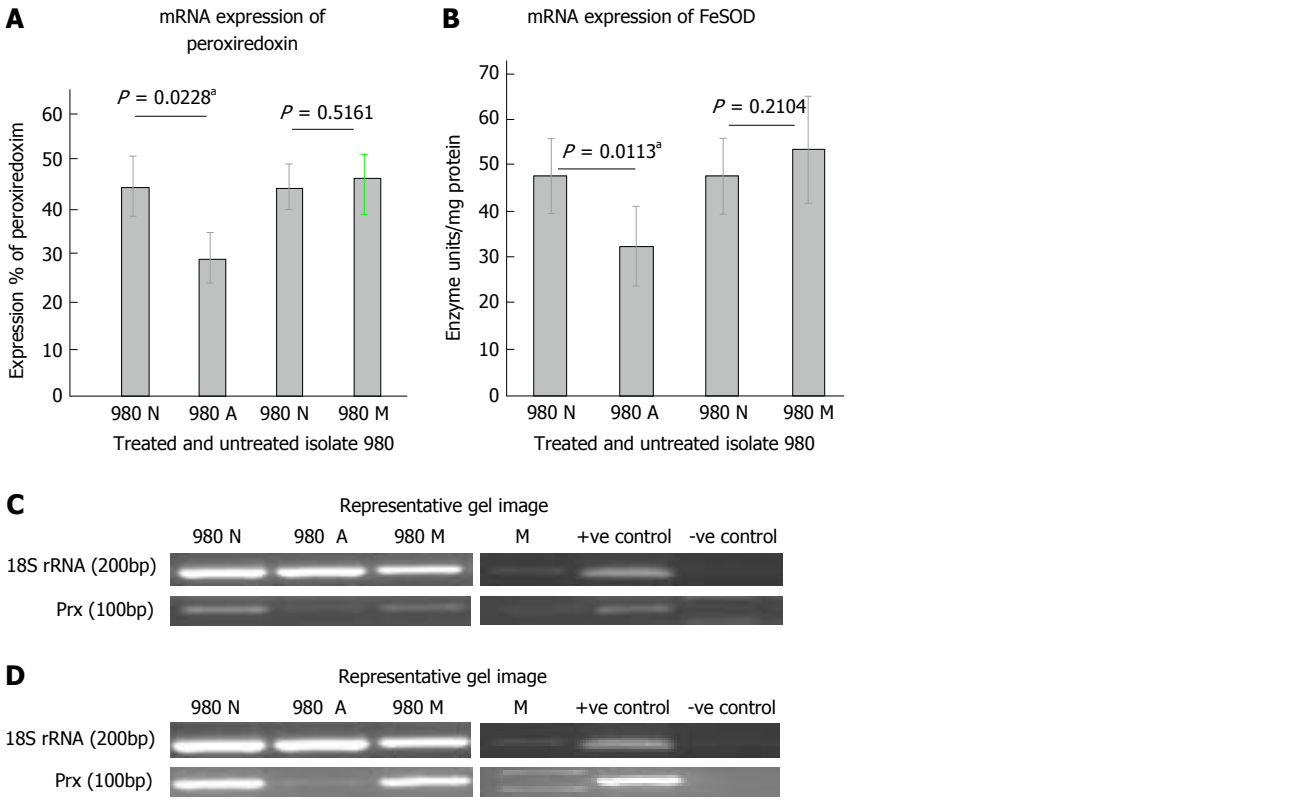

Figure 3 mRNA expression and activity levels of peroxiredoxin and FeSOD in clinical isolate 980 of *Entamoeba histolytica* during tatment with antiamoebic drugs. A: Graphical representation of densitometric data from semiquantitative RT-PCR analysis of Peroxiredoxin in clinical isolate 980, untreated (980 N) and after auranofin (980 A) and metronidazole treatment (980 M). Densitometric values are expressed as percent after normalizing with 18S rRNA. Each pair of columns show the data for untreated and treated isolate. Data are mean ± SE for three independent experiments. In case of auranofin treatment ($^aP < 0.05$); B: Graphical representation of densitometric data from semiquantitative RT-PCR analysis of FeSOD in clinical isolate 980. Densitometric values are expressed as percent after normalizing with 18S rRNA. Each pair of columns show the data for untreated and treated isolate. Data are mean ± SE for three independent experiments. In case of auranofin treatment ($^aP < 0.05$); C: Representative gel pictures of peroxiredoxin expression. 980 N: Untreated isolate; 980 A: Auranofin treated; 980 M: Metronidazole treated; M: Marker; +ve C: HM-1: IMSS cDNA used as +ve control; -ve C: cDNA from blank medim used as -ve control; D: Representative gel pictures of FeSOD expression. 989 N: Untreated isolate; 989 A: Auranofin treated; 989 M: Metronidazole treated; M: Marker; +ve C: HM-1: IMSS cDNA used as +ve control; -ve C: cDNA from blank medim used as -ve control.

of TrxR. We also observed similar results in two clinical isolates of *E. histolytica* 654 and MS96 (Dhaka) after treatment with metronidazole for 24 h[16].

When Auranofin (0.5 μmol/L) treatment was given for 24 h to isolate 989, it gave an increase in thioredoxin reductase expression at the mRNA level though not significant and a similar increase at the protein level, which was significant. The downregulation of TrxR expression was not observed in this isolate perhaps due to the low concentration of auranofin used. In case of mRNA expression of peroxiredoxin and superoxide dismutase there was no significant change on treatment of isolate 989 with 0.5 μmol/L auranofin for 24 h, compared to untreated controls. At this concentration of auranofin perhaps the toxic effects of the drug were not seen, and therefore the detoxifying enzymes were not upregulated.

On treatment of isolate 989 with 20 μmol/L metronidazole, the thioredoxin activity showed slight upregulation though not significant, at the mRNA level and a significant upregulation at the protein level. Thioredoxin reductase reduces metronidazole to its active form along with two other NADPH dependent oxidoreductases[6]. An upregulation of this enzyme could therefore explain the higher sensitivity of 989 to metronidazole. Tazreiter *et al*[17] also reported a modest upregulation of Thioredoxin reductase when an *E. histolytica* population was exposed for 8 h to a concentration of 50 μmol/L of metronidazole. On the other hand, TrxR, peroxiredoxin and FeSOD were shown to be downregulated at the protein level in *E. histolytica* when treated with 50 μmol/L metronidazole for up to 8 h[3]. However, our results with clinical isolates 989 did not show any significant change in peroxiredoxin or superoxide dismutase activity. We are yet to fully decipher the contribution of thioredoxin reductase in clinical isolates of Entamoeba during metronidazole stress.

Clinical isolates of *E. histolytica* from Delhi show different tolerance to antiamoebic drugs metronidazole and auranofin. Isolate 980 shows a higher tolerance to metronidazole with MIC's of 80 μmol/L compared to other clinical isolates studied. In the isolate 980, mRNA expression levels of thioredoxin reductase, FeSOD and Peroxiredoxin were downregulated with auranofin treatment. TrxR enzyme activity also showed an inhibition at the protein level. Our results further confirm that in clinical isolates auranofin acts by inhibition of TrxR and perhaps the treated isolate has a lower capacity to combat

oxidative stress as is evident by the downregulation of FeSOD and Peroxiredoxin. Metronidazole treatment also inhibited the mRNA expression of Thioredoxin reductase and the TrxR enzyme activity showing a higher tolerance to metronidazole. Lack of metronidazole activation could be the reason for the increase in tolerance of isolate 980 to metronidazole.

Isolate 989 showed a greater sensitivity to metronidazole compared to other clinical isolates, with MIC's of 30 μmol/L. However, upon treatment with auranofin at 0.5 μmol/L, we could not observe the toxic effect the drug. Treatment of 989 with metronidazole, showed an upregulation of TrxR activity indicating higher rate of conversion of the drug to it active form.

We conclude that each clinical isolate responds differently to drug stress. Infections of *E. histolytica* which show a greater metronidazole tolerance can be effectively combated by treatment with auranofin.

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COMMENTS

Background

Entamoeba histolytica (*E. histolytica*) infections are endemic to India and are associated with a high rate of morbidity and mortality. Metronidazole has been in use for around more than 40 years for treating amoebiasis. It has been used against both bacterial and protozoan infections.

Research frontiers

Widespread use of this drug and short term exposure of the parasite to sub lethal doses has been the reason for the development of metronidazole tolerance by the parasite. This raises concerns on the treatment of amoebiasis. To combat this problem, auranofin a gold containing drug which is already in use against rheumatoid arthritis was tested for activity against entamoeba infections and found to be very effective.

Innovations and breakthroughs

The authors studied the effect of auranofin in two clinical isolates of Entamoeba from New Delhi which showed different tolerance to metronidazole. This research shows that on antioxidant profiling at mRNA level and at enzyme activity level, there is a difference in the expression of antioxidant enzymes between the isolates showing different tolerance. Till today no work has been done on the effect of Auranofin and the changes in antioxidant enzyme activities on clinical isolates of *E. histolytica* upon treatment with antiamoebic drugs.

Applications

Auranofin has been found to be effective in clinical isolates of *E. histolytica*, which was highly tolerant to metronidazole. This is a very important finding, and since auranofin is a drug already in use in humans, it can be safely used as an alternative therapy against amoebic infections.

Terminology

Minimum inhibitory concentrations: In microbiology, minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial drug that will inhibit the visible growth of a microorganism after overnight incubation. In medicine, culturing the organism infecting a patient with available antibiotic drugs and determining the MICs, is important for identifying the correct drug dosage to administer to the patient. Drug tolerance: The ability of an organism to persist despite the presence of high concentrations of drug which normally inhibit their growth.

Peer-review

It is interesting with regard to drug resistance.

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CASE REPORT

Disseminated cryptosporidiosis: Case report and literature review

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Author contributions: Khalil S, Mirdha BR and Paul J designed the report; Khalil S, Panda A and Singh Y performed experiments; Khalil S, Panda A and Singh Y collected the patient's clinical data; Khalil S, Mirdha BR and Paul J analyzed the data and wrote the paper.

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Institutional review board statement: The study was approved by Institutional Ethics Committee of All India Institute of Medical Sciences, New Delhi, India.

Informed consent statement: All the participants were apprised about the study protocol. During the meetings, enrolled individuals (guardians/parents in case of children) were informed that their participation is voluntary and they have all the rights to withdraw from the study at any time without giving any reason.

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Abstract

Cryptosporidiosis, better known as an intestinal disease may disseminate to infect other sites including the respiratory tract. Little information however is available on respiratory cryptosporidiosis that may largely be due to lower frequency of respiratory cryptosporidiosis. Respiratory cryptosporidiosis has been majorly reported in immunocompromised individuals and children. Here we report a case of respiratory and intestinal cryptosporidiosis in a fifteen months old child with CD8+ deficiency. The patient in spite of treatment with Nitazoxanide and Azithromycin followed by Intravenous immunoglobulin and Bovine colostrum had a fatal outcome. The *Cryptosporidium* spp. isolate was subjected to molecular characterization. The *Cryptosporidium* spp. was identified both in stool specimen and Endotracheal aspirate (ETA). The blood sample was negative for *Cryptosporidium* spp. The *Cryptosporidium* spp. isolate from stool as well as ETA was identified as *Cryptosporidium hominis* (*C. hominis*) using Multiplex Allele Specific Polymerase Chain Reaction assay and was subtyped as IaA23G1R1 subtype using *gp60* gene polymerase chain reaction assay followed by sequencing.

Key words: Cryptosporidiosis; Disseminated disease; CD8+ deficiency; *Cryptosporidium hominis*; Subtyping

Core tip: Disseminated cryptosporidiosis has rarely been reported because of the lower frequency as compared to intestinal cryptosporidiosis. Here we describe a case of patient who developed intestinal cryptosporidiosis followed by respiratory cryptosporidiosis. The *Cryptosporidium* isolate was identified as *Cryptosporidium hominis* subtype IaA23R2.

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INTRODUCTION

Cryptosporidium species are globally important enteric protozoan parasites with infection most commonly observed in immunocompromised individuals and children[1]. It is mainly presented as diarrheal disease leading to significant morbidity and mortality in developing countries especially the rural areas[2,3]. *Cryptosporidium* is one of the leading causes of infectious diarrhea in Indian children with prevalence ranging from 1.1%-18.9%[4].

In immunocompetent individuals cryptosporidial diarrhea is transient self-limiting illness[5]. Infections amongst immunocompromised individuals may also become extra-intestinal, spreading to other sites including the gall bladder, biliary tract, pancreas and pulmonary system[5] and possible dissemination may occur through haematogenous route as post-mortem observation has shown the presence of *Cryptosporidium* spp. in the lumen of sub-mucosal colonic blood vessels[6]. Respiratory cryptosporidiosis can occur in immunocompetent children suffering from cryptosporidial diarrhea with unexplained cough[7]. In humans, it was first reported in 1984 in a patient with symptoms of chronic cough, fever, tachypnea, dyspnea with chest radiographs, findings consistent with interstitial pneumonia[8]. Several other cases of respiratory cryptosporidiosis have been reported albeit the relative rarity of the disease. It is postulated that involvement of the respiratory tract may result in transmission of *Cryptosporidium* oocysts by aerosols and fomites.

Present report describes the detection, identification and subtyping of a *Cryptosporidium* spp. detected in the respiratory secretions [Endotracheal aspirate (ETA)] in a fifteen months old child with CD8+ immunodeficiency. Genus specific *18S rRNA* gene polymerase chain reaction (PCR) assay was used to detect *Cryptosporidium* spp., where as Multiplex Allele Specific (MAS) PCR assay was used to identify the species of *Cryptosporidium.* The *gp60* gene was targeted for PCR assay followed by sequencing for subtyping.

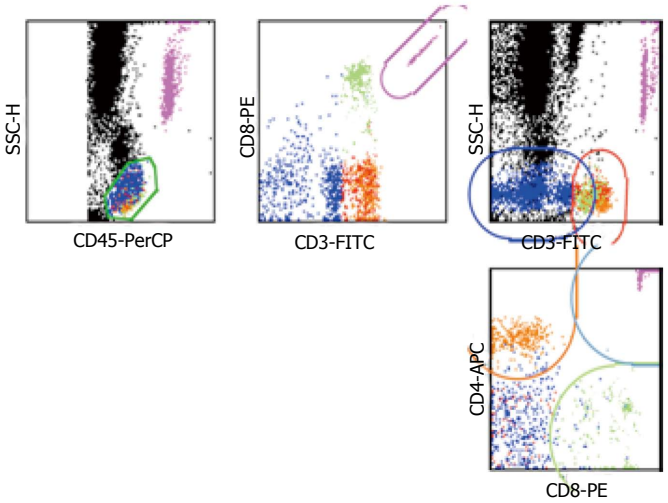
CASE REPORT

A fifteen months old male child with the complaints of fever and rapid breathing for at least two weeks along with cough and vomiting was admitted to the pediatric in-patient department of our tertiary care hospital. Patient had a history of recurrent fever since two and half months with each episode lasting for 10-15 d with an intermittent non-febrile stage of nearly a week. Child had decreased appetite and had lost approximately 500 g of body weight within three months. On admission the child was emaciated and severely malnourished. The patient was fourth child to a non consanguineous couple and was a follow up case of disseminated Cytomegalovirus (CMV) infection, periodic neutropenia, and Iron deficiency anaemia with CD8+ immunodeficiency. The CD4+ and CD8+ counts of child are given in Figure 1.

The patient's main clinical and laboratory findings on admission were as follows: Tachycardia (166/min), Tachypnea (52/min), fever (99 ℃), severe anaemia (6.8 g/dL), neutropenia [Total Leukocyte Count (3600/μL); Neutrophils (40%)] and normal Platelet count (5.25×10^5/μL). Serum biochemicals revealed normal kidney function [blood urea (15 mg/dL), Creatinine (0.2 mg/dL)]. Deranged Serum Glutamic Oxaloacetic Transaminase (99 IU) and elevated Alkaline phosphatase (565 IU) were observed. Serum immunoglobulin levels were normal (IgG-1137 mg/dL, IgA-108 mg/dL, IgM 102 mg/dL). Anthropometric measurements revealed Z-scores less than 3 [head circumference (41.5 cm); body weight (4.6 kg) and height (61 cm)] suggesting severe malnutrition. During present admission child was given prophylaxis of Fluconazole (25 mg once daily), Co-trimoxazole (20 mg/kg per day), lactose free diet as well as anti reflux measures. Urine and blood samples were sent for microbiological investigations and treatment for Severe Acute Malnutrition was started.

Urine culture was positive for *Escherichia coli* (> 10^5 CFU/mL) sensitive to Amikacin/Nitrofurantoin/Zosyn. Blood culture did not show growth of any pathogenic organism. Patient was started with combination of Injection (Inj) Piperacillin and Tazobactam 470 mg IV thrice a day along with Inj Amikacin 75 mg OD, Inj Vit K 2 mg, Syp cetrizine 2.5 mL (OD), Tab lanzol (Lansoprazole) 5 mg OD. In addition, Inj Magnesium sulphate 1 mL OD, Syp Zinc 2.5 mL OD, Folic acid tablets 5 mg OD then 1 mg OD, Syp Calcium carbonate and vitamin D3 2.5 mL OD, syp Atoz Multivitamin 2.5 mL OD along with the prophylaxis of Co-trimoxazole and Fluconazole.

On third day of admission patient was afebrile, there was no vomiting and was accepting the oral feed well, however, he subsequently developed diarrhea with a frequency of up to 20 stools in a day. Domperidone and Cinnarizine combination syrup at a dosage of 1 mL thrice daily was started and urine and blood samples were again sent for microbiological investigations along with the stool sample. All the clinical samples after all the microbiological investigations were negative for any pathogens except the stool sample that showed high load of *Cryptosporidium* spp. Oocysts, *i.e.*, upto 30 oocysts present per high power field. Syrup Nitazoxanide (NTZ) at a dose of 100



CD3+ %Lymphocytes	40%
CD3+ Absolute Count	948
CD3+CD8+ %Lymphocytes	4%
CD3+CD8+ Absolute Count	97
CD3+CD4+ %Lymphocytes	25%
CD3+CD4+ Absolute Count	595
CD3+CD4+CD8+ %Lymphocytes	0%
CD3+CD4+CD8+ Absolute Count	1
CD45+ Absolute count	2378

Figure 1 Immunological profile of the patient with CD8+ deficiency.

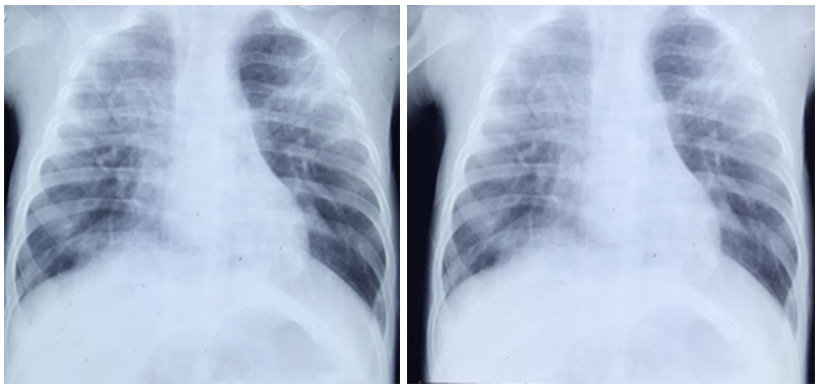


Figure 2 Chest X-ray of CD8+ deficient child showing bilateral infiltrates.

mg/5 mL twice daily and Azithromycin (AZ) at a dose of 45 mg/20 mL in normal Saline was given intravenously. However, diarrhea did not improve even after a week of continuous NTZ and AZ treatment. To circumvent this unresolving cryptosporidial diarrhea, Intravenous Immunoglobulin and Bovine Colostrum were started along with NTZ and AZ. After 24-48 h of this treatment there was no improvement in diarrheal symptoms and child began to develop respiratory distress with tachypnea and cool peripheries and further worsening. Chest X-ray showed bilateral infiltrates (Figure 2). Several causal possibilities of bilateral pneumonia were considered that included reactivation of CMV infection, *Pneumocystis jirovecii* Pneumonia, respiratory cryptosporidiosis as well as fungal sepsis. Amphotericin B was added for treatment of fungal sepsis and Co-trimoxazole dose was increased. On tenth day child had further worsening with increased heart rate (128/min), respiratory rate of 100/min along with increased frequency of voluminous diarrhea. Child was intubated and ventilated. Stool, endotracheal aspirate and blood samples were further sent for investigation with a special reference to detect presence of *Cryptosporidium* spp. oocysts. Child showed no signs of improvement and died. The primary reason leading to death was ascribed to disseminated cryptosporidiosis, with antecedent causes including immunodeficiency, periodic neutropenia and disseminated CMV infection.

The samples of stool, blood and ETA received in our laboratory were subjected to molecular analyses using primers given in Table 1. DNA was extracted from stool sample using QIAmp DNA stool minikit (Qiagen, United States) and blood and ETA using QIAmp Easy Blood and Tissue minikit (Qiagen, United States). The DNA from these three samples was subjected to diagnostic PCR assay using genus specific *18S rRNA* gene primers. For identification of species Multiplex Allele Specific PCR assay targeting *DHFR* gene was used. For subtyping *GP60* gene was targeted. Gel based extraction of the PCR products was performed as per the manufacturer's instructions using MinElute gel extraction kit (Qiagen, United States). Sequencing for the study isolates was performed in both forward and reverse direction on ABI 3500xL Genetic Analyzer from Chromous Biotech. Consensus sequences were pairwise aligned using Clustal W and were manually refined using the BioEdit program version 7.0.4.

A band size of 435 bp was obtained from DNA extracted both from stool sample as well as ETA showing

Table 1 Primers used in the study

Gene (Ref.)	Primers	Amplicon size
18S rRNA[9]	CPB-DIAGF: 5′AGCTCGTAGTTGGATTTCTG-3′ CPB-DIAGR: 5′-TAAGGTGCTGAAGGAGTAAGG-3′	435 bp
MAS PCR[10]	CINF: 5′GTGGGGATTTAACTTGATTT 3′	575 bp
	CINR: 5′GGTATTTCTGGGAAATAAGT3′	357 bp
	1R: 5′GCTGGAGGAAATAACGACAATTA3′	190 bp
	2R: 5′TGTCCGTTAATTCCTATTCCTCTA3′	
GP60[11]	F1: 5′-ATAGTCTCCGCTGTATTC-3′	800-850 bp
	R1: 5′-GGAAGGAACGATGTATCT-3′	
	F2: 5′-TCCGCTGTATTCTCAGCC-3′	
	R2: 5′ GCAGAGGAACCAGCATC-3′	

the presence of *Cryptosporidium* spp. oocysts in both the samples. However, there was no amplification of cryptosporidial DNA from blood sample. MAS-PCR assay showed amplification of DNA bands suggestive of *Cryptosporidium hominis* (*C. hominis*). The desired band was obtained using *gp60* gene based PCR assay and the amplified products were sequenced. The sequences of *gp60* gene from both the sample identified them as Ia subtype family and IaA23G1R1 subtype. The sequence was submitted to genbank under Accession number KU169227.

DISCUSSION

Cryptosporidium spp. affects mainly the small intestines but infections of hepatic ducts, lungs and conjunctiva has also been reported[12,13]. However, a few case reports of respiratory cryptosporidiosis in human immunodeficiency virus (HIV)/AIDS cases are available[13]. Respiratory cryptosporidiosis is mostly presented as cough, dyspnea, low fever and abnormal chest X-ray with interstitial pneumopathy[14], with an unknown pathogenesis[15].

Respiratory route of *Cryptosporidium* transmission was suggested as results of epidemiological studies in children presumed to be immunocompetent. In a study from Switzerland, children with cryptosporidial diarrhea were more likely to have respiratory symptoms compared to those who had other infections, suggesting that respiratory infection may be common but transient in healthy individuals[16]. In a study from rural Brazil and Bangladesh, 50% and 33% of children with intestinal cryptosporidiosis had unexplained respiratory symptoms, respectively[17,18]. In a report from Gaza, 50% of children with cryptosporidial diarrhea and 10% of children without cryptosporidial diarrhea had respiratory symptoms and were also shedding *Cryptosporidium* in feces[19]. These findings led to the speculation that the respiratory system may serve as a viable alternative for *Cryptosporidium* propagation, transmission, and diagnosis, with or without apparent respiratory symptoms. Kumar *et al*[20], (2016) reported disseminated cryptosporidiosis in a 35 year old immunocompetent patient in India which was successfully treated with nitazoxanide.

In addition human respiratory cryptosporidiosis has been observed in patients with compromised cellular immunity as well as in individuals with induced immunosuppression; hence an association between cryptosporidiosis and depleted CD4+ T-cell count was established[6,15,21]. Disseminated cryptosporidiosis was reported in a child with nephrotic syndrome receiving immunosuppression[15].

In the present case, intestinal cryptosporidiosis was followed by respiratory cryptosporidiosis. Earlier studies have shown disseminated cryptosporidiosis originating from the intestinal tract infection. Subsequently cases of respiratory cryptosporidiosis lacking evidence of primary gastrointestinal involvement suggest the possibility of respiratory transmission of cryptosporidiosis[13,14]. The pathogenesis of *Cryptosporidium* spp. lung infection is still unclear. Infection can result from the inhalation of oocysts after vomiting or the hematogenous spread of the oocysts. Although intestinal *Cryptosporidium* spp. organisms are not usually invasive, oocysts have been found inside macrophages, which can have defective phagocyte killing ability[22]. In fact, *Cryptosporidium* spp. organisms can multiply in macrophages *in vitro*[23], suggesting that extraintestinal parasites might spread *via* circulating phagocytes. Regardless of the route of infection patients with disseminated cryptosporidiosis experience fulminant disease, fail to respond to existing therapies and have fatal outcome.

Human health risk is often compounded because there is only one Food and Drug Administration approved therapeutic agent, *i.e.*, NTZ. It reduces the duration of diarrhea and oocyst shedding in both immunocompetent and immunocompromised[24,25]. The patient was initially treated with nitazoxanide, however no improvement was seen in diarrhea and was later started with the combination therapy. Higher doses and longer duration of therapy may be needed for HIV-positive malnourished children to derive benefit from the drug[25]. Spiramycin, Azithromycin and Immunoglubulins have not been efficacious in controlled trials in patients with AIDS[26].

Isolate of *Cryptosporidium* spp. in our study was identified as *Cryptosporidium hominis* (*C. hominis*). Mercado *et al*[14], (2007) had isolated *C. hominis* from the respiratory secretions of an HIV sero-positive patient. No reports are available on the subtypes of *Cryptosporidium* spp. causing disseminated infection and/or infection of

the tissues other than intestinal[11].

Substantial information about which species and subtypes of *Cryptosporidium* infect humans and the pathogenic patterns of each of these is needed. *C. hominis* have the capacity to adapt to diverse environments and infect gastrointestinal as well as respiratory tract. This report supports the role of *C. homnis* as a human pathogen and the need to evaluate the importance of respiratory cryptosporidiosis as a disease in children as well as in immunocompromised host.

COMMENTS

Case characteristics

Fever, vomiting, cough and rapid breathing since 15 d and subsequent diarrhea.

Clinical diagnosis

Interstitial Pneumonia.

Differential diagnosis

Cytomegalovirus reactivation, *Pneumocystis* pneumonia, Fungal sepsis.

Laboratory diagnosis

Severe anaemia, neutropenia. Normal kidney fuction with deranged Serum Glutamate Oxaloacetic Transaminase and Alkaline phosphatase. Urine culture was positive for *E. coli*. Stool samples and Endotracheal aspirate were positive for *Cryptosporidium* species using PCR.

Imaging diagnosis

Bilateral infiltrates were seen on chest X-ray.

Treatment

Syp Nitazoxanide and Azithromycin along with Intravenous Ig and Bovine Colostrum were given to treat cryptosporidiosis.

Related reports

Mercado *et al* (2007) had isolated *C. hominis* from the respiratory secretions of an human immunodeficiency virus sero-positive patient. No reports are available on the subtypes of *Cryptosporidium* spp. causing disseminated infection and/or infection of the tissues other than intestinal.

Experience and lessons

Dissemination of cryptosporidiosis should be considered in patients with compromised cellular immunity as well as in individuals with induced immunosuppression.

Peer-review

It is a well written case report describing a 15 mo old child with CD8+ immunodeficiency, suffering from disseminated Cryptosporidiosis leading to death.

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Abstract

AIM

To assess healthcare seeking trends among Pakistani children with acute respiratory infections through comparative analysis between demographic health surveys (DHS) 2006-2007 and 2012-2013.

METHODS

Data of the last born children 0-24 mo of age of the sampled households from both the DHS was analyzed

after seeking permission from the DHS open access website. These were children who had suffered from cough and/or breathing difficulty in the past two weeks and sought health care thereafter. The trends of health care seeking were determined separately for the individual, household and community level according to the study parameters. χ^2 test was applied to compare these trends. A *P*-value of < 0.05 was considered significant.

RESULTS

Out of 2508 children in 2006-2007 there were 1590 with acute respiratory infections (ARI) according to case definition along with 2142 out of 3419 children in 2012-2013 DHS, whose data was analyzed. During 2006-2007, 69% cases sought healthcare for ARI which improved to 79% in 2012-2013. Additionally, it was revealed that when compared between 2006-2007 and 2012-2013, improvement in care seeking practices was observed among illiterate mothers (64% *vs* 77%) although there was minimal change in those literate. Similarly, those women working also showed an increase in healthcare seeking from 67% to 79%. Additionally, those belonging to low and middle socioeconomic class showed a marked increase as compared to those in the higher class where there was no significant change. Whereas those living in rural communities also showed an increase from 66% to 78%.

CONCLUSION

Increasing health budget, improving maternal education and strengthening multi-sectoral coordination are among the effective strategies to improve outcomes associated with healthcare seeking in ARI.

Key words: Acute respiratory infections; Demographic health surveys; Comparison; Pneumonia; Healthcare seeking

Core tip: Acute respiratory infections (ARI) contribute to childhood morbidity and mortality due to poor healthcare seeking among other causes. We aimed to identify the healthcare seeking trends among Pakistani children with ARI through comparative analysis between DHS 2006-2007 and 2012-2013. Data of last born children 0-24 mo was analyzed. In 2006-2007, 69% cases sought healthcare which improved to 79% in 2012-2013. Improvement was observed among poor, illiterate mothers, those working, and/or living in rural communities. It is therefore, important to develop strategies and interventions focusing on this category of caretakers to improve the outcome associated with ARI.

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INTRODUCTION

Acute respiratory infections (ARI), in general, and pneumonia, in particular, continue to be the leading causes of childhood morbidity and mortality worldwide[1]. This leads to a substantial burden on the healthcare system and can cause serious complications leading to economic and psychological burden at the household level[1,2]. According to the World Health Organization (WHO) and United Nations International Children's Emergency Fund, globally, over 2 million children die each year due to ARI with pneumonia[3] contributing to about one fifth of deaths of children aged less than five years[4]. Although numerous efforts have been put forth by the global community to combat these infections yet there has been little improvement witnessed in the reduction of the incidence of these diseases globally[2]. In Pakistan more than 250000 children die each year due to pneumonia[5,6]. This makes Pakistan enlisted among the top five countries globally with the highest childhood mortality due to pneumonia, a preventable disease[3,7]. Over 80% of these deaths occur due to lack of adequate and timely healthcare seeking[2]. It is, therefore, important to seek appropriate health care timely when children develop signs of ARI which include cough accompanied by short rapid breathing[8].

Studies have documented that delayed health care seeking behavior due to lack of knowledge regarding level of seriousness of the disease are among the leading causes[9-11]. In a formative research conducted in Nepal, Bangladesh and Pakistan regarding care seeking practices in the rural communities for new-born care, it was found that danger signs were not comprehended by the caretakers, therefore they mostly sought treatment from the local healers, *i.e*., homeopaths or non-qualified practitioners. Further, the traditional beliefs of influence of evil spirits, lack of understanding and awareness regarding the disease, distance from healthcare facilities, and high cost of treatment are hurdles in healthcare seeking behavior[12]. In another study conducted in Nigeria regarding the care seeking practices of childhood illnesses, 62% of the cases sought care beyond 24 h after the initiation of illness with 57% dealt with at home[13]. Another study conducted in Nigeria determining the effects socio-demographic characteristics on care seeking trends showed that high socioeconomic class and high maternal education were the major factors contributing towards early health care seeking from health facility[14]. In Pakistan, a study conducted to assess the healthcare seeking patterns in diarrhea among uneducated mothers of low resource peri urban Karachi showed that lack of transport and high cost of therapy are the major reasons for not seeking healthcare[15]. In a similar study

conducted in rural Ecuador, it was found that lack of recognition of the danger signs of acute respiratory illnesses led to delayed care seeking irrespective of the socioeconomic status[16].

It is, therefore, important to understand the factors leading to poor health seeking behaviors among the caretakers of children suffering from ARI. This can assist in developing effective strategies to improve survival of children under five of developing countries. ARI continues to be among the major health problems in Pakistan, especially when there has been limited evidence generated regarding the health care seeking behavior over the past decade. We aim to assess the trends of the health care seeking behaviors among caretakers of children with ARI especially pneumonia in Pakistan with respect to the demographic health surveys (DHS) conducted during 2006-2007 and 2012-2013.

MATERIALS AND METHODS

Study definitions

Acute respiratory illnesses: The cases of ARI were screened on the basis of children having cough, fast breathing or difficult breathing during the past two weeks according to the DHS questionnaire. These illnesses were divided into those who had "pneumonia" and those with "no pneumonia". Those cases which only had symptoms of cough and fever were labeled as "no pneumonia". Those cases which had fast breathing or difficult breathing due to involvement in the chest were labeled as "pneumonia".

Care seeking: Care seeking was defined as when the caretaker sought advice or treatment for the illness from any source according to the DHS questionnaire. This included all types of care sought, either traditional or formal. It included whether visiting a government or private hospital, private clinics or doctors, Lady Health Workers, homeopath doctor or other primary level healthcare settings. The study outcome was dichotomized as the one who sought care and the one who did not seek care for ARI.

Data source: It is retrospective study whereby secondary data from datasets of 2006-2007 and 2012-2013 Pakistan demographic health survey (PDHS), carried out by the National Institute of Population Studies, was utilized after seeking permission from the Demographic Health Survey Program under USAID. The survey in both the cases was designed to provide information on maternal and child health. The sampling methodology employed in both the surveys was multistage stratified cluster sampling whereby urban and rural samples were drawn separately. The sample was nationally representative in line with the population distribution in each province of the country. Random household sampling was conducted to select the respondents for the survey. Considering this is a sub analysis of an existing dataset therefore consent from the participants of the survey was not sought as the National Institute of Population studies had taken prior consent upon completion of the survey. The data set was downloaded from the public access website (http://www.measuredhs.com). In the 2006-2007 survey 10023 respondents were surveyed whereas in the 2012-2013 survey the sample was of 12943 respondents. The response rate for the 2006-2007 survey was 94.5% whereas that of 2012-2013 was 93.1%. The data was inspected for quality, completeness of information and comparability of variables required for the present analysis. The variables from the data set were then selected according to the objectives and the files were constructed. We selected lastborn children from 0-24 mo of age at the time of the survey who had suffered from cough in the last two weeks and were living with respondents/mothers. There were 2508 cases identified with history of cough in DHS 2006-2007 whereas 2012-2013 had 3419 such cases. According to the case definition 1590 and 2142 children in DHS 2006-2007 and 2012-2013 respectively with acute respiratory symptoms were finally analyzed.

Statistical analysis

The data was analyzed using STATA 10.0 software. Frequency and percentages were calculated for ARI and its care seeking. The trends of health care seeking were determined separately for the individual, household and community level according to the study parameters. The variables included maternal age, maternal education, working status, father's occupation, child age, gender, residence, place of delivery, delivery conducted by, socioeconomic status and geographical region. These variables were then coded and categorized. χ^2 test was applied to compare the trends of the rates of care seeking among the different categories according to the study parameters. A *P*-value of < 0.05 was considered significant. The statistical methods of this study were reviewed by the biostatistician of Shaheed Zulfiqar Ali Bhutto Medical University, Islamabad, Pakistan.

RESULTS

There were 1590 children with the respiratory symptoms identified during the DHS 2006-2007, of which 1167 (73.3%) had pneumonia and 374 (23.5%) cases had no pneumonia according to case definition. On the other hand, during DHS 2012-2013 there were 2142 children with respiratory symptoms among which 1610 (75.1%) were with pneumonia and 474 (22%) were with no pneumonia. The underlying diagnosis of the rest of the cases in both the surveys was unknown. According to the DHS data, health care for ARI was sought by 1108 (69.6%) cases in 2006-2007 which included 813 pneumonia cases and 295 no pneumonia cases. Similarly 1707 (79.7%) children sought health care in 2012-2013 with 1276 pneumonia and 431 no pneumonia cases (Table 1 and Figure 1).

The majority of ARI cases presented during

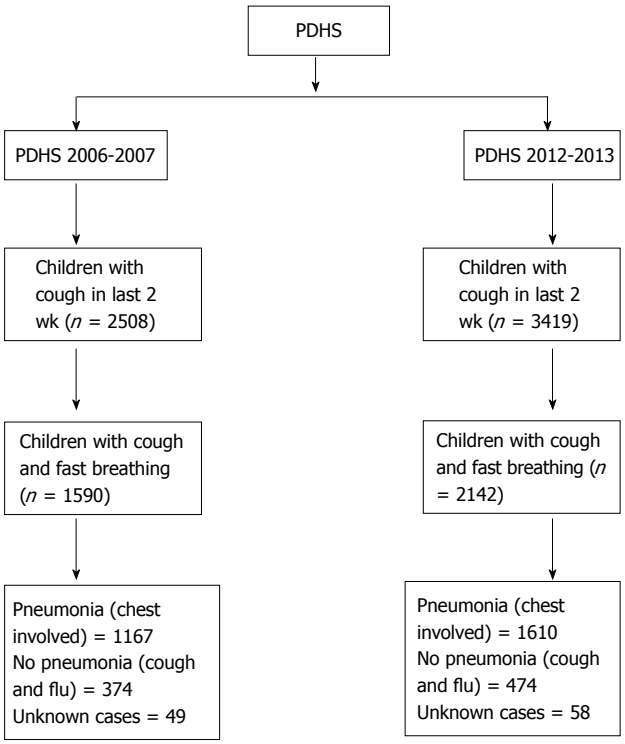

Figure 1 Distribution of children according to different acute respiratory infections categories. PDHS: Pakistan demographic and health survey.

Table 1 Prevalence of acute respiratory infections and its care seeking according to Pakistan demographic and health survey data 2006-2007 and 2012-2013, *n* (%)

	PDHS 2006-2007 *n* = 1590	PDHS 2012-2013 *n* = 2142
Pneumonia	1167 (73.3)	1610 (75.1)
No pneumonia	423 (26.7)	532 (24.8)
Care seeking for ARI		
Sought	1108 (69.6)	1707 (79.7)
Not sought	482 (30.3)	435 (20.3)

PDHS: Pakistan demographic and health survey; ARI: Acute respiratory infections.

Table 2 Baseline characteristics of children with acute respiratory infections according to Pakistan demographic and health survey data 2006-2007 and 2012-2013, *n* (%)

	PDHS 2006-2007 *n* = 1590	PDHS 2012-2013 *n* = 2142
Age		
Neonates (up to 28 d)	377 (23.6)	452 (21.0)
1 to 6 mo	1213 (76.3)	1690 (78.9)
Gender		
Male	877 (55.2)	1143 (53.4)
Female	713 (44.7)	999 (46.5)

PDHS: Pakistan demographic and health survey.

2006-2007 and 2012-2013 were infants aged 1 to 6 mo, *i.e.*, 1213 (76.3%) and 1690 (78.9%) respectively followed by the neonates (< 1 mo). Additionally, male gender was predominantly affected as revealed from both the surveys (Table 2).

According to the statistics, as majority of the cases of ARI are usually diagnosed as pneumonia, therefore further analysis of cases of pneumonia was conducted[17]. According to the DHS 2006-2007, mothers of younger age group (up to 20 years) were more likely (77.1%) to seek care for their child with pneumonia as compared to the other age groups, *i.e.*, 66.9%. Whereas according to the DHS 2012-2013 the mothers aged up to 30 years were found more likely (82.0%) to seek care as compared to 77.3% of other age groups.

There was increase in healthcare seeking among the illiterate men and women in 2012-2013 as compared to 2006-2007. This rise in healthcare seeking observed among illiterate women was from 64% to 77% whereas that for men was from 59% to 77% between 2006-2007 to 2012-2013. However, the trend in educated caretakers remained static when the average of those with primary, secondary and above educational status was compared in both surveys. It was, however, found that of all the levels of education, those who were educated sought care with a higher proportion as compared to those illiterate.

In DHS 2006-2007 it was noted that the jobless fathers and the ones involved in unskilled work were less likely to seek health care, however, in the DHS survey 2012-2013 fathers' occupation had no influence on variability of care seeking. Similarly, it was observed that health care seeking was increased among working women from 2006-2007 to 2012-2013 but was decreased among the non working women from 2006-2007 to 2012-2013 as shown in Table 3.

In 2006-2007, it was found that children delivered at healthcare facilities sought healthcare more than those delivered at home (76.7% *vs* 65.5%). Those who were delivered by health professionals also sought healthcare more as compared to those born by traditional birth attendants (79.0% *vs* 58.4%) and the ones delivered by c-section were more likely to seek care during an ARI episode as compared to ones delivered normally (82.2% *vs* 68.5%). The same findings were observed for data analyzed in 2012-2013 as shown in Table 3. When data of all these variables was compared between 2006-2007 and 2012-2013 it was found that there was a rise in seeking healthcare in 2012-2013 as shown in Table 3.

It was noted that from 2006-2007 to 2012-2013, there was a rise in care seeking among those in lower (60% *vs* 75%) and middle socioeconomic class (69% *vs* 82%) whereas that for the higher class remained almost static (82.4% *vs* 84.9%).

Similarly, caretakers living in urban areas as compared to rural (77.5% *vs* 66.0%) were more likely to seek care in 2006-2007 with a similar trend in 2012-2013 (82% *vs* 78%). However, it was noted that those living in rural communities improved care seeking from 2006-2007 to 2012-2013 (66% *vs* 78%).

It was found that the care seeking behavior

Table 3 Trends of rates of pneumonia care seeking according to Pakistan demographic and health survey data 2006-2007 and 2012-2013, *n* (%)

	PDHS 2006-2007			PDHS 2012-2013		
	Care seeking (*n* = 813)	No care seeking (*n* = 354)	*P* value	Care seeking (*n* = 1276)	No care seeking (*n* = 325)	*P* value
Individual level factors						
Maternal age (yr)						
Up to 20	37 (77.1)	11 (22.9)	0.01	43 (75.4)	14 (24.5)	0.02
20 to 30	421 (71.5)	167 (28.5)		685 (82.0)	150 (18.0)	
31 or above	355 (66.9)	176 (33.1)		548 (77.3)	161 (22.7)	
Maternal education						
Illiterate	515 (64.2)	287 (35.7)	< 0.001	700 (77.1)	208 (22.9)	0.007
Primary	136 (78.6)	37 (21.4)		221 (79.7)	56 (20.2)	
Secondary or above	162 (84.4)	30 (15.6)		355 (85.3)	61 (14.6)	
Father's education						
Illiterate	252 (59.5)	171 (40.4)	< 0.001	390 (77.2)	115 (22.7)	0.004
Primary	151 (74.7)	51 (25.2)		182 (75.8)	59 (24.2)	
Secondary or above	405 (75.7)	130 (24.3)		704 (82.5)	151 (17.5)	
Father's occupation						
Skilled manual	149 (77.2)	44 (22.8)	< 0.001	214 (84.2)	40 (15.7)	0.11
Unskilled manual	168 (65.3)	89 (34.6)		431 (78.1)	121 (21.9)	
Professional/ technical	63 (72.4)	24 (27.5)		123 (78.8)	33 (21.2)	
Agriculture related	151 (62.1)	92 (37.9)		150 (81.1)	35 (18.9)	
Services/clerical	253 (75.9)	80 (24.1)		319 (79.9)	80 (20.1)	
Did not work	25 (50.0)	25 (50.0)		33 (70.2)	14 (29.7)	
Others	4 (100.0)	0 (0.0)		6 (75.0)	2 (25.0)	
Maternal working status						
Working	182 (67.1)	89 (32.8)	0.3	247 (79.4)	64 (20.6)	0.46
Not working	631 (70.4)	265 (29.5)		1029 (80.1)	261 (20.3)	
Age of child (mo)						
Neonates	205 (74.2)	71 (25.7)	0.03	275 (81.1)	170 (19.8)	0.13
1 to 2	351 (71.8)	138 (28.2)		590 (81.3)	135 (18.7)	
3-6	257 (64.0)	145 (36.0)		411 (76.5)	126 (23.5)	
Gender						
Male	456 (70.7)	189 (29.3)	0.39	686 (80.1)	170 (19.8)	0.63
Female	357 (68.3)	165 (31.6)		590 (79.1)	155 (20.8)	
Place of delivery						
Healthcare facility	326 (76.7)	99 (23.3)	< 0.001	683 (83.4)	135 (16.6)	0.008
Home	485 (65.5)	255 (34.5)		593 (75.7)	190 (24.3)	
Delivery conducted by						
Health professional	331 (79.0)	88 (21.0)	< 0.001	672 (82.2)	146 (17.9)	0.01
Trained birth attendant	247 (71.1)	100 (28.9)		343 (79.5)	89 (20.7)	
Other untrained attendant	229 (58.4)	163 (41.6)		261 (74.5)	89 (25.4)	
Delivery by cesarean section						
Yes	79 (82.2)	17 (17.7)	< 0.001	169 (84.5)	31 (15.5)	0.19
No	734 (68.5)	337 (31.4)		1107 (79.1)	293 (20.9)	
Household level factors						
Socioeconomic status						
Lower class	326 (60.2)	216 (39.8)	< 0.001	524 (75.3)	171 (24.7)	0.005
Middle class	154 (69.3)	68 (30.7)		279 (82.3)	60 (23.6)	
Higher class	333 (82.6)	70 (17.4)		473 (84.9)	94 (15.1)	
Community level factors						
Residence						
Urban	287 (77.5)	83 (22.5)	< 0.001	531 (82.1)	116 (17.9)	0.05
Rural	526 (66.0)	271 (34.0)		745 (78.1)	209 (21.9)	
Geographical region						
Punjab	321 (73.4)	116 (26.5)	< 0.001	401 (87.3)	58 (12.6)	< 0.001
Sindh	332 (78.8)	89 (21.2)		244 (82.4)	52 (17.5)	
KPK	144 (51.4)	136 (48.5)		335 (70.9)	137 (29.0)	
Balochistan	16 (55.1)	13 (44.8)		129 (72.8)	48 (27.1)	
Gilgit Baltistan	-	-		114 (84.4)	21 (15.6)	
Islamabad (ICT)	-	-		53 (85.4)	9 (14.5)	

PDHS: Pakistan demographic and health survey; KPK: Khyber Pakhtun Khwah.

improved among the caretakers belonging to the provinces of Punjab, Khyber Pakhtun Khwah (KPK) and Baluchistan in 2012-2013 as compared to 2006-2007. Additionally, it was found that is people living in

Punjab and Sindh were more likely to seek care than those living in Balochistan and KPK provinces (73.4% and 78.8% *vs* 51.4% and 55.1% respectively) in 2006-2007. A similar trend was observed in 2012-2013 survey and details given in Table 3.

DISCUSSION

Our results show that there has been a significant improvement in health care seeking behavior among caretakers in some of the variables from 2006-2007 to 2012-2013. These included illiterate mothers and fathers, working caretakers, children who were born in the healthcare facilities, belonging to middle and lower socioeconomic class and those living in rural community.

The increase in the healthcare seeking among illiterate mothers and fathers could be attributed to two factors. One reason could be the increase in the migration of large proportion of rural population, the majority of which comprises of illiterate individuals, for higher employment and better healthcare opportunities[18]. Evidence suggests that urban residence provides better opportunities for healthcare seeking as indicated by a study conducted by Kugelman *et al*[18], who identified this difference in behavior among urban and rural communities. They indicated that individuals in rural communities had a lower weekly financial budget not only for healthcare but also for transport to a healthcare facility which led to delayed or no healthcare seeking[19]. Another study conducted in Bangladesh, whereby secondary data analysis of their DHS was conducted also revealed that urban population sought more care than rural one[20]. Another reason could be improvement of health management systems at the provincial level down to the level of the district and greater financial autonomy based on needs after devolution of the health ministry in 2011 whereby the autonomy for implementation of policies/programs was given to the provinces[21,22]. This benefitted the people in the form of quality healthcare delivery close to their doorstep[23]. This might also have led to increased health care seeking practice among the children who were born in health facilities[24].

Since the healthcare seeking behavior was found to be raised among the working women, it could be attributed to the provision of opportunity in terms of more fiscal space and awareness regarding utilization of health services. This points to improvement in woman's autonomy which has been observed to more among working women[25]. Autonomy is defined as the woman's ability to act freely and independently. With increase in both formal and informal employment among Pakistani women, they tend to make decisions based on their independent will[26]. This could be one of the major factors which might have contributed towards improved healthcare seeking among this group of women. This point is also reflected by decrease in healthcare seeking by non-working women. A study conducted in India on healthcare seeking behavior for antenatal care revealed that non-working women were less likely to seek healthcare as compared to working ones[27].

Results also show that there was a difference of healthcare seeking among the provinces with caretakers in Punjab and Sindh seeking more health care as compared to Balochistan and KPK. This could also be the result of the devolution due to which the provinces might be moving at a difference pace from each other in terms of implementation of the health programs[22]. It has been observed that the province of Punjab has been in the forefront due to their emphasis on the health as compared to the other provinces[22]. If there are some common factors, other provinces can learn from the experience of that province and improve the indicators however, due to geographical SEC and cultural and political variations should be taken into account while doing so.

It was also evident that when compared among the various socioeconomic classes in both surveys, healthcare seeking behavior was improved more in the lower and middle socioeconomic class as compared to the upper class. This again could be attributed to a change in the health systems which have become more accessible at the grass root level. In addition to that as the per capita income of the country has slightly improved therefore the individuals belonging to this class have shown this increase in healthcare seeking trend[25]. Another reason could increase in the educational level of the middle class women of the society which again points towards their autonomy as indicated earlier

In order to develop strategic policies and programs, it is important that information related to healthcare seeking behaviors and factors determining these behaviors are utilized. The socio demographic context of these behaviors is among the most impactful of all[28]. It is, therefore, important to develop strategies and interventions to promote appropriate and timely health care seeking behavior for children as in the absence of such interventions, there is a strong likelihood that there will further be an increase for the vulnerable population to suffer even more. Benefits of improving healthcare seeking are tremendous especially in settings where public health services are limited.

Based on these findings the following recommendations are made to influence policy and practice for improving care seeking for children with ARI: Educating the mothers especially in the rural communities and those of the older age group, on picking up the signs of ARI early through community based integrated programs; improving of quality of care and awareness so that care seeking is sought within 24 h of initiation of symptoms; more investment on infrastructure, by increasing the annual health budget, in the public sector making it accessible for the poor community which cannot afford the private sector; multi sectoral approach by enhancing and strengthening coordination among education, planning, health, and communication at the

planning and implementation phases for example by developing safety net programs which could in turn improve the socioeconomic class of the community; More research on the consequences of delay in health care seeking practices and behavior, to decrease or prevent the high costs of illness.

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COMMENTS

Background

Acute respiratory infections particularly pneumonia continue to be the leading killer in under five children in Pakistan, among other developing countries. Poor healthcare seeking practices are a major predisposing factor towards this. There is little evidence whereby trends of healthcare seeking for pneumonia have been assessed over a specific period of time. The authors have, therefore assessed the trends of healthcare seeking for pneumonia from data obtained from two consecutive demographic health surveys (DHS) (2006-2007 and 2012-2013) to determine any differences therein and to suggest suitable recommendations to improve the system.

Research frontiers

It is important to determine trends in healthcare seeking for pneumonia patients to aid in development of effective innovative strategies. Demographic characteristics play an impactful role over healthcare seeking behavior which has not been assessed to that extent in Pakistan, let alone compared between various surveys.

Innovations and breakthroughs

This is the first study to have compared data between two consecutive DHS to assess healthcare seeking trends for children with pneumonia in Pakistan.

Applications

Healthcare seeking trends tend to affect the outcome of pneumonia. It is important for clinical practitioners to understand what factors are contributing to the fatal outcomes in terms of healthcare seeking so that they could undergo tailor made management of patients based on clinical findings. And this is the kind of information which this study has provided. Various strategies can be suggested for different categories of caretakers. Considering majority of cases did not seek healthcare due to lack of knowledge as indicated in this study, therefore illiterate mothers can be educated by developing and using validated standardized tools. Similarly, when a child is born in a facility the mother can be made aware of the potential diseases including pneumonia at the fist point of contact of the newborn to the physician. One suggestion could be the linking of these awareness sessions with the standardized immunization program whereby the caretaker's knowledge is reinforced every time the child is immunized by the healthcare provider. Similarly, the vaccination cards could have printed pictorial messages indicating prevention and identification of the signs of pneumonia. If a patient comes from a poor socioeconomic class, the practitioners should counsel the caretakers upon the preventive measures of pneumonia development to avoid further episodes or new episodes in other children of the household. Additionally, those practitioners who are involved at the policy level should emphasize on development of effective strategies to cater to the factors which needs emphasis. Similarly, the results of this study indicate that there is a gap at the community level which can be filled by regular training of the Lady Health Workers who can manage the early signs and refer the patients for hospital care timely. This can in turn also assist in documentation of cases of ARI coming right from the community level which otherwise is identified through DHS based on recall.

Peer-review

The basic study is well designed and written.

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CASE REPORT

Atypical manifestation of herpes esophagitis in an immunocompetent patient: Case report and literature review

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Abstract

Herpes simplex virus (HSV) is known to cause esophagitis in immunosuppressed patients; however, it is rarely seen in immunocompetent patients. We present a unique case of HSV esophagitis in a healthy male, without any immunocompromising conditions or significant comorbidities. The patient presented with a two-week history of dysphagia, odynophagia and epigastric pain. Physical exam revealed oral hyperemia without any visible ulcers or vesicles. He underwent esophagogastroduodenoscopy which noted severe esophagitis with ulceration. Esophageal biopsies were positive for HSV. Serology was positive for HSV as well. After initiating treatment with Famciclovir 250 mg 3 times/d, high dose proton pump inhibitor and sucralfate, patient had complete resolution of symptoms at his 2.5 wk follow up appointment. Subsequent workup did not reveal any underlying immune disorders. While HSV is a known causative of esophagitis in the immunocompromised, its presentation in healthy patients without any significant comorbidity is uncommon. Presentation with a systemic viral prodrome further makes this case unique.

Key words: Immunocompromised; Herpes; Esophagitis

Core tip: Herpes simplex virus (HSV) is known to cause esophagitis in immunosuppressed patients but rarely does it cause esophagitis in immunocompetent patients. We present a unique case of a healthy 43-year-old man who presented with two week course of dysphagia, odynophagia, and epigastric pain. Work up, which included esophagogastroduodenoscopy, revealed severe esophagitis with ulceration and biopsies showed HSV. He was successfully treated with famciclovir.

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INTRODUCTION

Herpes simplex virus (HSV) is a known causative agent for esophagitis in the immunocompromised host. However, its presence in immunocompetent and otherwise healthy patients is rare. Patients with esophagitis often presents with concerning clinical features such as odynophagia, dysphagia, and retrosternal pain. Endoscopic visualization demonstrates mid distal esophageal ulcers[1,2]. Our case report describes a unique case of HSV esophagitis in an immunocompetent, otherwise healthy male host with an unusual prodome of symptoms.

CASE REPORT

A 43-year-old man with a past medical history of migraines presented with a 10-d history of progressively worsening dysphagia, odynophagia and burning sensation at the epigastric region with radiation to the back. Although the patient denied any oral ulcers, his symptoms were exacerbated by oral intake lasting for several hours which lead to decreased oral intake, weight loss, generalized weakness, and fatigue. He denied any changes in his bowel habits or gastrointestinal blood loss.

The patient reported a recent history of severe chills, a pustule on his face with purulent discharge, diffuse body aches, nausea, vomiting, and arthralgias. He also noted a diffuse erythematous pruritic rash on his back, lower chest and abdomen that worsened over the last week prior to presentation.

Patient was born in North Carolina and had lived in Florida for over 10 years. He denied any known allergies and denied starting any new medications including over the counter medicines or herbal medications. He reported being employed as a state trooper and denied any sick contacts or recent travel. He admitted to a 20-year history of daily alcohol use. Denied any history of smoking or illicit drug use. He did endorse having an affair with a new girlfriend with whom he had oral sex prior to onset of his symptoms.

Physical examination revealed a man in no acute distress. Mild hyperemia was noted in the oropharynx without any lesions on the mucosal membranes. The patient was afebrile, acyanotic, and anicteric. Other systems were unremarkable. No cervical, axillary, or inguinal lymphadenopathy was noted. Skin exam revealed erythematous follicular rash on chest, back and lower abdomen. No insect bites or target rash was noted. Basic labs, including complete blood count and complete chemistry, were unremarkable. He underwent diagnostic testing which resulted in a positive Hepatitis A IgG. Hepatitis B, hepatitis C, human immunodeficiency virus (HIV), monospot, Lyme disease and rapid plasma reagin testing were negative. On complete blood count (CBC), he had white blood cell count of 7.7 with atypical lymphocytes that were reported at 8% of total, which was slightly higher than normal. HSV 1/2 antibody was positive. Blood cultures and urine cultures remained negative. Esophagogastroduodenoscopy (EGD) revealed severe esophagitis with evidence of erosions and superficial bleeding in the mid esophagus (Figures 1 and 2).

Pathology on esophageal biopsy demonstrated mild chronic inflammation with reactive changes and focal intestinal metaplasia along with positive immunohistochemical staining for HSV (Figures 3 and 4). Also, it revealed multinucleated squamous cells with margination of chromatin and molding of nuclei. Stains for cytomegalovirus (CMV) were negative and Grocott methenamine silver stain did not reveal any fungal organisms. Unfortunately, PCR for HSV-1 and 2 on biopsy specimen was reported as HSV-1/2 and no distinction could be made as to whether HSV virus in biopsy specimen was type 1 or 2. The final diagnosis on pathology report for esophageal biopsy was "HSV esophagitis with ulceration".

Treatment with famciclovir 250 mg thrice daily, pantoprazole 40 mg daily and sucralfate was begun. Within three days of treatment initiation, patient felt well enough to be discharged home on the same regimen. He was seen at a follow up visit after two and a half weeks and reported remarkable improvement of symptoms and was able to tolerate all foods with subsequent weight gain.

DISCUSSION

HSV esophagitis is a common pathologic agent in immunocompromised patients who are on chronic immunosuppressive medications as well as patients with underlying neoplasia, organ transplant recipients and HIV infection. Infection in an immunocompetent host has been noted in rare cases and may be secondary to a primary infection or reactivation of a latent infection[3].

These infections are mostly caused by HSV-1. HSV-2

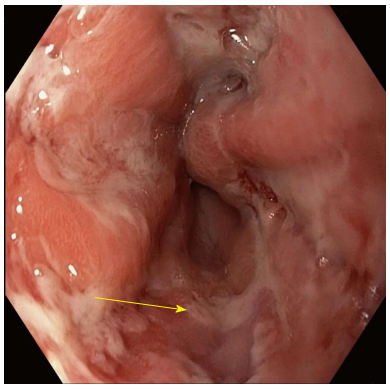

Figure 1 Diffuse ulcerations in the mid esophagus with inflammatory exudate, severe esophagitis 32-42 cm in esophagus.

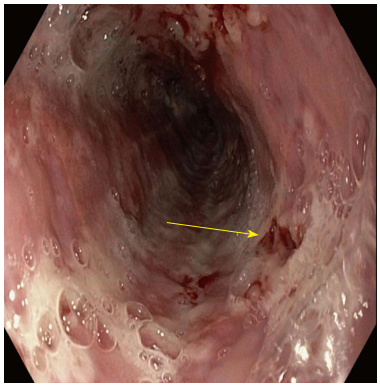

Figure 2 Endoscopic appearance of shallow base ulcers with evidence of erosions and superficial bleeding of the esophagus.

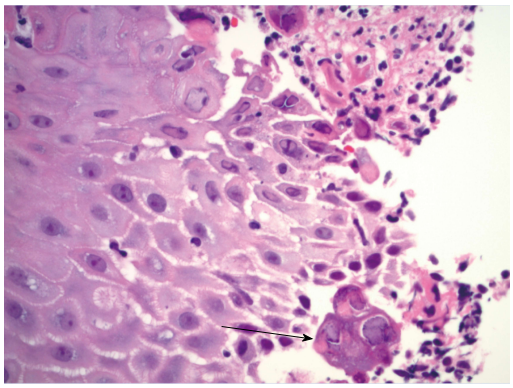

Figure 3 Hematoxylin and Eosin stain showing squamous cells with viral cytopathic effects of herpes simplex virus 1 and 2 - cowdry type A inclusion body- which includes multinucleation, margination of chromatin and molding of nuclei.

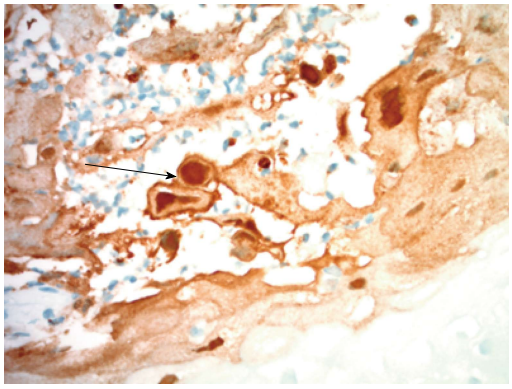

Figure 4 Immunohistochemistry stain for herpes simplex virus 1 and 2 shows the involved nuclei as staining brown for herpes simplex virus viral particles.

related esophagitis is extremely rare. Traditionally, the first episode of oral-facial HSV infection is caused by HSV-1 with a current rise in oral HSV-2 infections paralleling the change in sexual behaviors[2]. Also, HSV-1 is becoming a predominant cause of first episode of genital herpes in young adults[2].

Oral-facial HSV infections caused by either HSV-1 or HSV-2 can be transmitted to heterosexual partner involved in an orogenital contact. With an increasing prevalence of unprotected orogenital sexual contacts, there has been a reported increase in transmission of anogenital infections caused by HSV to the oropharynx resulting in HSV related esophagitis[2].

We believe that our patient who was recently involved in an orogenital contact with a new girlfriend and may have contracted HSV-1 or HSV-2 infection resulting in infection of his oropharynx by either HSV strain and eventually causing esophagitis.

We performed a review of all available literature on the topic and noted a paucity of data. Patients usually present with typical symptoms of dysphagia, odynophagia, retrosternal discomfort, heartburn, nausea, vomiting, weight loss, cough, and epigastric pain[3]. Once noninfectious etiologies of esophagitis have been ruled out, the most common etiologies include HSV, CMV and Candida. Other etiologies should be considered in differential diagnosis of esophagitis and these include but are not limited to malignancy, esophageal stricture, reflux esophagitis and eosinophilic esophagitis. HSV is the second most common cause of infectious esophagitis[4]. It has been described in a wide age range and appears to have a pattern of male predominance with a ratio of 3:1[1]. Prior exposure to HSV is reported in 20% of cases[4]. Symptoms are usually characterized by acute onset of odynophagia and epigastric pain in the majority of patients with or without prodromal symptoms. These prodromal symptoms usually consist of fever, malaise, pharyngitis, and upper respiratory symptoms[5]. Oropharyngeal manifestations (herpes labialis) may be associated and has been reported in 20% of cases[5].

The distal esophagus is the most common site involved (63.8%) and usually affects with the large portions of esophageal mucosa (92.1%)[5]. In the early stages of the disease, discrete vesicles can be appreciated; these typically slough off to form circumscribed ulcers. The mucosa is friable and inflamed in most cases (84%) and can be covered with a white exudate. Late stages are characterized by mucosal necrosis[4]. Biopsies from the edge of ulcers can confirm the disease and such

biopsies usually show cowdry type A inclusion bodies which are very classic for HSV related infections. Although polymerase chain reaction (PCR) is preferred for its high sensitivity (92%-100%) and specificity (100%), cell culture and virus isolation are considered the gold standard[1,3]. Direct immunofluorescence assays can be applied as a faster diagnostic tool but is limited by a sensitivity of only 69%-88%[1]. Serology is usually a poor diagnostic tool considering most adults have prior exposure to HSV, however, it can be useful in cases of primary HSV were patients experience seroconversion[4]. Although, not usually obtained, double contrast radiographic studies can visualize superficial ulcers against a background of normal mucosa[6]. Ulcers develop a punctate, stellate, or ring like configuration and may be surrounded by radiolucent mounds of edema.

The diffuse rash in our patient was of uncertain etiology. HSV may present with dermatologic manifestations in the form of erythema multiforme[7]. This is an immune mediated hypersensitivity reaction and in the setting of HSV, presents with oral lesions and a classic targetoid rash and typically resolves with HSV treatment. HSV esophagitis in an immunocompetent host is usually self-limiting and recurrence is rare. Rare complications such as upper GI bleed and perforation of the distal esophagus can occur without treatment[5].

Benefits of therapy have no clear evidence, although it has been shown to shorten duration of illness by about 6 d[4]. Due to odynophagia, IV Acyclovir is traditionally the drug of choice which can be then transitioned to oral prodrugs such as famciclovir and valaciclovir. Cases treated with acyclovir showed clinical response in less than 3 d with complete resolution of symptoms within 4-14 d of therapy[1]. Our case highlights the importance of keeping HSV esophagitis as one of the differential diagnosis for immunocompetent patients who present with symptoms suggestive of esophagitis.

COMMENTS

Case characteristics

A healthy 43-year-old man presented with 2 wk history of dysphagia, odynophagia, and epigastric pain radiating to back that worsened with oral intake.

Clinical diagnosis

Esophagogastroduodenoscopy (EGD) revealed severe esophagitis with evidence of erosions and superficial bleeding in the mild esophagus. Pathology on biopsy was positive for herpes simplex virus (HSV).

Differential diagnosis

With ulcerate lesions in the esophagus and odynophagia, differential is broad and includes infection, malignancy, esophageal strictures, reflux esophagitis, and eosinophilic esophagitis.

Laboratory diagnosis

Gold standard for diagnosis is cell culture and virus isolation but PCR is preferred for its high sensitivity and specificity.

Imaging diagnosis

Esophagogastroduodenoscopy provides direct visualization of the ulcerate lesions that characterize HSV esophagitis.

Pathological diagnosis

Ulcer biopsy can confirm the disease and they usually show cowdry type A inclusion bodies which are pathognomonic for HSV related infections.

Treatment

Therapy has shown to shorten duration of illness and options include intravenous acyclovir or oral famcyclovir and valacyclovir.

Related reports

Very few reports exist for immunocompetent patients with HSV esophagitis. It is more common in immunocompromised hosts.

Term explanation

EGD is abbreviation for esophagogastroduodenoscopy.

Experiences and lessons

HSV esophagitis must be kept in the differential when any patient presents with odynophagia regardless of immune status.

Peer-review

This manuscript presents an interesting case and it is well written, organized, and informative.

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World Journal of Clinical Infectious Diseases

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AIM AND SCOPE

World Journal of Clinical Infectious Diseases (*World J Clin Infect Dis*, *WJCID*, online ISSN 2220-3176, DOI: 10.5495) is a peer-reviewed open access (OA) academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

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CASE REPORT

Autoimmune hepatitis in human immunodeficiency virus infection: Case report and literature review

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Abstract

The infection due to human immunodeficiency virus (HIV) is characterized by the progressive reduction of $CD4^+$ T lymphocytes and the compromise of other cell lines of the immune system, resulting in immunosuppression. In this context, autoimmune diseases could be considered contradictory, however, cases of autoimmune diseases during this infection have been described, including autoimmune hepatitis (AIH), which is uncommon and has few case reports within medical literature, none of them from Latin America. In this case report where a patient with an HIV infection on combined antiretroviral treatment developed acute elevation of transaminases, hyperbilirubinemia, and deterioration in hepatic synthetic function. Although initially an antiretroviral drug-induced liver injury was suspected, during the study a diagnosis of autoimmune hepatitis was proven, which required treatment with corticosteroid and azathioprine, obtaining a satisfactory response and managing to continue the

antiretroviral therapy. Autoimmune diseases in HIV infection must be taken into account. In the case of hepatitis in patients with HIV on antiretroviral treatment, the differentiation between viral hepatitis caused by autoimmune diseases or medications is essential to establish an adequate treatment, and avoid the suspension of the antiretroviral therapy.

Key words: Autoimmunity; Autoimmune hepatitis; Human immunodeficiency virus; Anti-human immunodeficiency virus agents; Drug-induced liver injury

Core tip: In the combined antiretroviral therapy era, human immunodeficiency virus (HIV) infection can show diverse manifestations others than infections, been autoimmunity a paradoxical but well described phenomena in this scenario. The objective of this case report is to illustrate a rare condition as autoimmune hepatitis in HIV infected patients on therapy, with an additional literature review, to help clinicians in the approach of this disease and the differentiation with drug induced liver injury related to antiretroviral therapy.

Noreña I, Morantes-Caballero JA, Garcés A, Gómez BJ, Rodríguez G, Saavedra C, Otero W. Autoimmune hepatitis in human immunodeficiency virus infection: Case report and literature review. *World J Clin Infect Dis* 2017; 7(4): 50-57 Available from: URL: http://www.wjgnet.com/2220-3176/full/v7/i4/50.htm DOI: http://dx.doi.org/10.5495/wjcid.v7.i4.50

INTRODUCTION

The global incidence of infection due to human immunodeficiency virus (HIV) reached its peak in 1997. Since then it has remained relatively constant. However, the prevalence has increased as the survival rate has improved thanks to the combination antiretroviral therapy (cART)[1]. With these therapies there is a reduction of the mortality as well as the risk of developing severe events related to the acquired immune deficiency syndrome (AIDS) in 57% of cases, regardless of age, gender and $CD4^+$ T lymphocyte count[1,2], and constitutes the most effective strategy for preventing onwards HIV-1 infections[3]. The early onset of therapy results in better outcomes for patients, even with an advanced disease[2]. In 2%-18% of patients, treatment must be discontinued due to adverse effects, especially in the liver, preventing patients to benefit from this treatment[4]. Drug induced liver injury (DILI) manifests itself through hepatitis and an increase of the aminotransferases, making it indistinguishable from any other hepatitis[5].

HIV affects the $CD4^+$ lymphocytes and alters other cell lines of the innate immune system (macrophages, monocytes and dendritic cells)[6]. This can lead to autoimmune diseases[5-7]. So far, few cases have been published of HIV/AIDS patients with concomitant autoimmune diseases, such as vasculitis, systemic lupus erythematosus, psoriasis, Graves' disease, and less commonly, autoimmune hepatitis (AIH)[5-9]. After reviewing the literature, there were only 22 cases of autoimmune hepatitis described in patients with HIV, in the described cases the $CD4^+$ lymphocyte count was above 100 cells/mm^3, and was initially considered as liver toxicity caused as a side effect of the antiretroviral therapy[10-18]. Taking into account the little information on these two entities occurring simultaneously, the case of a patient with HIV, who, in the course of their disease, developed AIH, is presented.

CASE REPORT

A 26-year-old male, student by occupation, who due to a self-suspicion screening for syphilis was diagnosed with a 2-year HIV infection; he was infected by a former partner in an unprotected man-man relation. In addition, he had a history of intravenous drug use, and no family history was identified. The initial study found an HIV viral load of 179488 copies/mL and a $CD4^+$ T lymphocyte count of 298 cells/mm^3 and $CD8^+$ of 2067 cells/mm^3. The complete blood count and liver and kidney profiles were normal. The serology for hepatitis A, B and C were negative, as well as the tuberculin test and the serology for syphilis. No opportunistic infections were documented. Treatment was started using Efavirenz/Tenofovir disoproxil fumarate/Emtricitabine, having a good tolerance and achieving a lower HIV viral load and satisfactory $CD4^+$ T lymphocyte recovery. One year after starting the treatment, in a follow-up appointment clinical hypothyroidism of an autoimmune etiology was documented (anti-microsomal antibodies, anti-thyroglobulin and positive anti-thyroid peroxidase), and treatment with levothyroxine was started.

Several months later he was admitted to the emergency room due to jaundice. Hyperbilirubinemia (> 15 mg/dL) was found, with a predominance of direct bilirubin, severe elevation of transaminases (> 2000 IU/L), prolongation of the prothrombin time INR (1.95), and a discrete increase of alkaline phosphatase and gamma glutamyl transferase. Among other differential diagnostics, hepatotoxicity by cART was suspected, and this therapy was immediately discontinued. During hospitalization, the serology for hepatotropic viruses was negative (A, B, C and E), viral loads for virus B, C, Epstein Bar (EBV) and cytomegalovirus (CMV) were undetectable. The hepato-biliary ultrasound and portal doppler were normal. The antinuclear antibodies were positive 1:160 dils, with mottled pattern, and negative anti-mitochondrial and anti-muscle antibodies. High levels of immunoglobulin G were found. A liver biopsy was performed, which reported a lymphoplasmacytic inflammatory infiltration with eosinophils and severe interface activity, hepatocytes with peri-central inflammation and focal necrosis ("compatible with

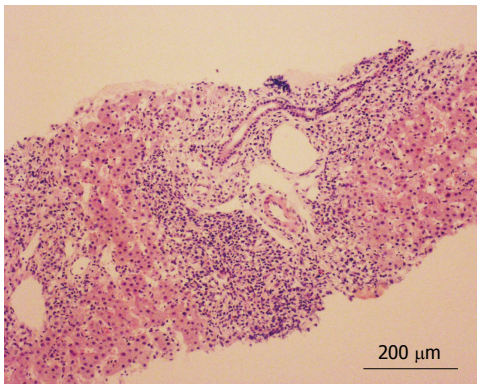

Figure 1 Histologic findings. Histological section, H and E staining (scale bar × 10). Liver fragment with the presence of abundant inflammatory lymphoplasmacytic infiltrate with eosinophils and severe interface activity.

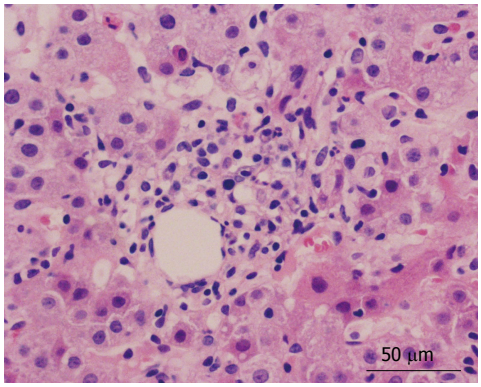

Figure 2 Histologic findings. Histological section H and E staining (scale bar x 40). Presence of lymphoplasmacytic infiltrate with eosinophils and hepatocytes with pericentral fibrosis inflammation.

autoimmune hepatitis") (Figures 1 and 2). Treatment with oral prednisolone 1 mg/kg per day was started, with a significant improvement and a quick normalization of amino transferases and bilirubin, and was discharged without restarting antiretroviral therapy.

As an outpatient, there was a gradual reduction of the prednisolone dose, until leaving a minimum dose of 10 mg/qd. With normal liver profile, cART treatment was restarted, replacing efavirenz with raltegravir and continuing Tenofovir disoproxil fumarate/Emtricitabine. A month later, the patient went again to the emergency room for recurrence of jaundice; increased serum transaminase levels greater than 2000 mg/dL were documented as well as bilirubin of 18 mg/dL at the expense of the direct bilirubin and the prolongation of prothrombin time and INR. He was hospitalized, cART therapy was once again suspended and doses of prednisolone of 1 mg/kg qd, were started, adding Azathioprine 25 mg/qd, and achieving a progressive reduction of transaminase and bilirubin. There was confusion to determine if the worsening of the hepatitis was due to the decrease of prednisolone (due to autoimmune hepatitis) or when the cART started (due to DILI). A Medical Board was held between the services of gastroenterology, internal medicine and

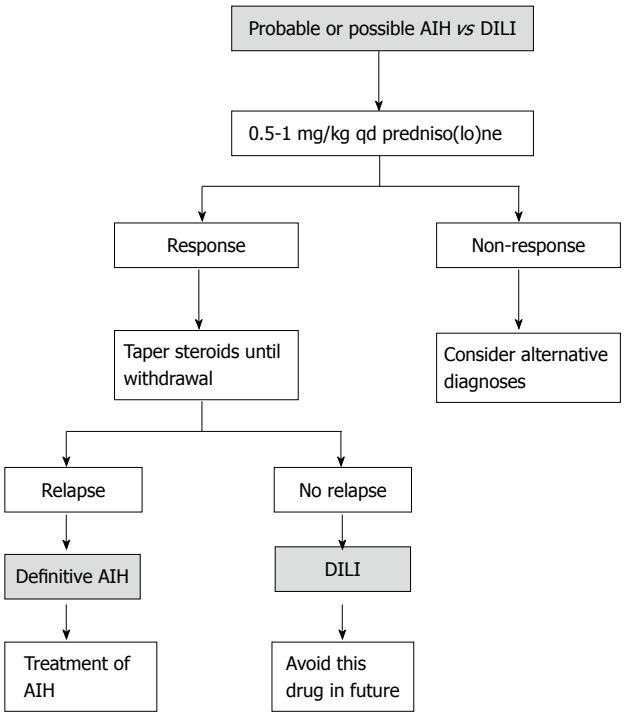

Figure 3 Diagnostic algorithm suggested. Diagnostic algorithm suggested to differentiate between autoimmune hepatitis (AIH) of drug-induced liver injury (DILI). Following the suspension of the steroid, a long-term follow-up is recommended (6 mo to 3 year) so as not to lose a late relapse of autoimmune hepatitis. Adapted from EASL Clinical Practice Guidelines: Autoimmune hepatitis, 2015.

infectious diseases, and based on the current guidelines for the diagnosis of autoimmune hepatitis and their differentiation with hepatitis due to medications (Table 1 and Figure 3), autoimmune hepatitis was defined as the definitive diagnosis[19]. To completely rule out DILI, in a hospital environment, antiretroviral therapy was restarted and the liver profile monitored, which continued to improve until it was normal and the patient discharged.

After six months of follow-up, the patient was asymptomatic, receiving maintenance therapy for AIH only with azathioprine 100 mg/qd, HIV treatment and replacement hormone therapy with levothyroxine. The liver profile has remained unchanged. Figure 4, shows the evolution of the liver profile before and during treatment.

DISCUSSION

A case of autoimmune hepatitis in a patient with HIV infection has been presented. The coexistence of these diseases is rare: Three independent reviewers conducted the literature search in different electronic databases (PubMed, Science Direct, EBSCO and ProQuest), the search terms used were a combination of keywords and MeSH terms and included "HIV", "AIDS", "Human immunodeficiency virus", "Autoimmune disease" "Autoimmunity", "Autoimmune Hepatitis", "Anti-HIV Agents", "Anti-Retroviral Therapy" and "Drug-

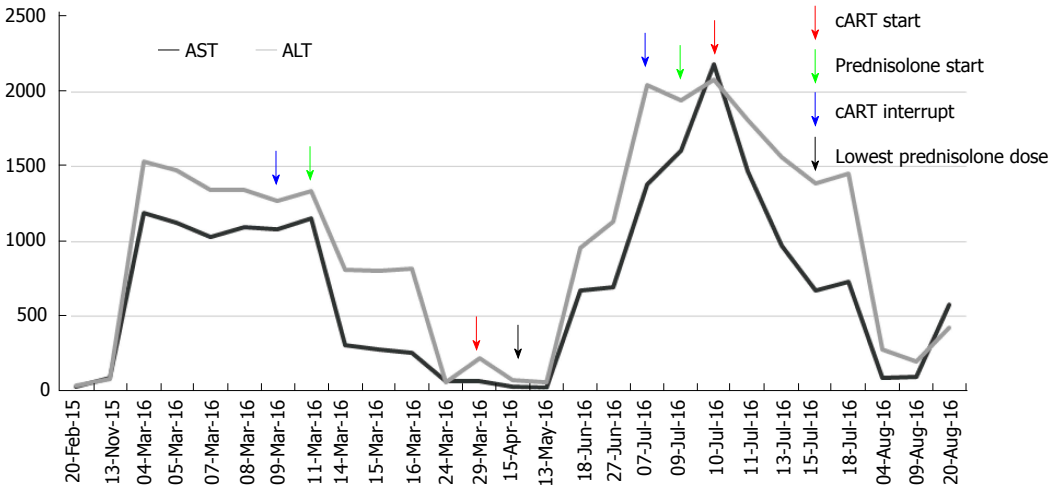

Figure 4 Transaminase values during follow-up. Aspartate transaminase (AST) and alanine transaminase (ALT) values (UI/L) and their relationship with the interventions carried out and date (DD/MM): At the start of the follow-up, the patient was already receiving antiretroviral therapy, in the first interventions there is some confusion that does not allow to differentiate whether the decrease in serum transaminase levels was due to the onset of immunosuppressive therapy or the interruption of the antiretroviral therapy; also there is no indication to show if the second peak elevation of transaminases was due to the resumption of the antiretroviral therapy or due to the decrease in the dose of immunosuppressive therapy. In the end, it may be noted that, although the antiretroviral therapy was reset, the decline of transaminases continued with the immunosuppressive therapy, which helped to confirm the diagnosis of autoimmune hepatitis. cART: Combination antiretroviral therapy.

Table 1 Simplified diagnostic criteria for autoimmune hepatitis, adapted from EASL Clinical Practice Guidelines: Autoimmune hepatitis, 2015

Parameter	Discriminator	Score
ANA or ASMA$^+$	⩾ 1:40	1
ANA or ASMA$^+$	⩾ 1:80	2
ANA or LKM$^+$	⩾ 1:40	2
ANA or SLA/LP$^+$	Any title	2
IgG or gamma globulins	> ULN	1
	> 1.1 × ULN	2
Liver biopsy	Compatible with AIH	1
	Typical AIH	2
	Atypical	0
Absence of viral hepatitis	No	0
	Yes	2

Definitive autoimmune hepatitis ⩾ 7; probable autoimmune hepatitis ⩾ 6. Typical liver biopsy for autoimmune hepatitis: Each one of the following characteristics must be present: Interface hepatitis, lymphocytic/lymphoplasmacytic infiltrates in the portal tracts, and extending toward the lobes, emperipolesis (active penetration by a cell within another cell), hepatocyte rosettes formations. Liver biopsy consistent with autoimmune hepatitis: Chronic hepatitis with lymphoplasmacytic infiltrate without all the characteristics considered typical. Atypical liver biopsy: Signs of another diagnosis. ANA: Anti-nuclear antibodies; ASMA: Anti-smooth muscle antibodies; LKM: Liver kidney microsomal antibodies; SLA/LP: Antibodies to soluble liver antigen/pancreas; IgG: Immunoglobulin G; ULN: Upper limit of normal; AIH: Autoimmune hepatitis.

Induced Liver Injury" in the title, abstract, or keywords with no limit in dates, types of publication or language. Nine reports with only 22 cases of patients with both entities were found, none of them described in Latin America[10-18]; their characteristics can be seen in Table 2. However, in patients with HIV, AIH can be under-diagnosed because of the complexity of the diagnosis and the confounding factors present in patients with HIV (adverse effects to medications, hidden viral hepatitis, opportunistic infections, neoplasms)[14,16,20].

The exact mechanisms by which patients with HIV may present AIH are unknown. However, an HIV infection is related to various autoimmunity phenomena[5,9]. The direct damage caused by the virus, the molecular mimicry, the deregulation of T/B cells, the generation of immune complexes and auto-antibodies can trigger damage to its own tissues[8,21]. AIH stands out due to the presence of autoreactive CD4$^+$ and CD8$^+$ T lymphocytes, and a cellular response mediated by antibodies with a particular increase in the Th17 subtype and IL17 release. This can be silent in stages of deep immunosuppression, with similar clinical and paraclinical reactivations similar to the immune reconstitution inflammatory syndrome (IRIS-like) that occurs with the immunological recovery associated with the effective antiretroviral therapy[18]. Four immunological stages have been described in HIV infection[9,10], the acute infection-latency (stage Ⅰ-Ⅱ) stage and the immune restoration related to antiretroviral therapy (stage Ⅳ) are the most likely to develop autoimmune disorders, furthermore, some disorders mediated by CD8$^+$ cytotoxicity are present in the AIDS phase (stage Ⅲ)[8].

Based on the hypotheses set forth, it is considered that, in the case described above, the immune restoration by antiretroviral therapy, in addition to the predisposition to autoimmune disorders, were the triggers of the hepatitis, the thrombocytopenia, and Hashimoto's thyroiditis. This is consistent with the reports described in literature that supports the association between AIH and other autoimmune diseases, mainly Hashimoto's thyroiditis[14].

The AIH is a disease common in women, however, it can occur in males, as has been reported in other

Table 2 Clinical characteristics of the cases reported

Author	Vasilios	Wan	Daas	Coriat	Puius	O'Leary	Hagel	Cazanave	Murunga
Patients reported (*n*)	1	4	1	1	3	1	1	1	9
Age (min/max) (yr)	38	49-56	42	48	29-65	44	52	43	23-45
Gender (M/F)	M	1-3	F	F	1-3	F	M	F	1-8
$CD4^+$ cell count (cells/μL) (min/max)	216	174-357	157	250	200-259	269	641	500	253-876
HIV viral load at presentation (min/max) (copies/mL)	81000	< 50-232734	120000	No data	< 75-8687	< 50	UD	< 1000	UD-8
AST (min/max) (IU/L)	120	45-186	1526	No data	20-500	NO DATA	343	No data	13-34
ALT (min/max) (IU/L)	274	45-167	777	355	12-641	26-940	192	500	10-39
cART	Z, L, Nelfinavir	No data	F, T, Etravirine	R, E, Atazanavir	L (2), Z, E (3), F (1), T (1), stavudine	T, F, E	Didanosine, stavudine, E	L,Stavudine, E	E (7) F (6) T (8) L (3) N (1) Z (1) L/R (1)
Positive autoantibodies (*n*) dils	ANA 1:320 ASMA 1:40	ANA 1:20-40 ASMA(1)	ANA: 1:1280 ASMA(-)	ANA: 1:80 LKM: 1:320	ANA (2) 1:80-640 ASMA (1)	ANA 1:160	ANA: 1:2560	ANA: 1:8000 ASMA: 1: 4000	ANA (4), ASMA (6), ALKM (1)
Inmunoglobulin G (g/L)	29.25	639-2020	46	30	3050-7500	2640	39	Normal	16.5-55.2
Fibrosis	No	No	Yes	No	Yes	No	No	Yes	Yes
International AIH group score (min/max)	15	10-18	19	No data	10-15	22	15	No data	12-20
Treatment	No data	Prednisolone (4) Azathioprine (3)	Prednisone	Prednisolone	Prednisolone, Azathioprine	Prednisolone, Azathioprine	Prednisolone	Prednisolone, Azathioprine	Prednisolone
Publication Year	2005	2009	2011	2008	2008	2008	2012	2006	2016
Ref.	[10]	[11]	[12]	[13]	[14]	[15]	[16]	[17]	[18]

M: Male; F: Female; UD: Undetectable; ARV: Antiretroviral therapy; L: Lamivudine; E: Efavirenz; F: Emtricitabine (FTC); L/R: Lopinavir/Ritonavir; T: Tenofovir disoproxil fumarate; N: Nevirapine; Z: Zidovudine; ANA: Anti-nuclear antibodies; ASMA: Anti-smooth muscle antibodies; ALKM: Anti-liver kidney microsomal antibodies.

cases (Table 2), and is characterized by an injury with hepatocellular pattern, circulating autoantibodies (anti-nuclear, anti-smooth muscle, or anti-microsomal liver-kidney), elevated levels of immunoglobulin G and a consistent liver biopsy. Histologically, there are characteristic changes (non pathognomonic) that include interface hepatitis, plasmacytic infiltration and regenerative hepatocyte rosettes[14].

The diagnosis is established by combining criteria and discarding other entities that cause hepatitis (Table 2)[17,19]. The acute case this patient developed occurs only in 25% of AIH, and makes diagnosis difficult, being that the negativity of the anti-smooth muscle antibodies is frequent[17]. In this patient, the diagnosis of AIH was achieved by the combination of clinical, paraclinical and histological findings, the good response to immunomodulatory therapy and the exclusion of other causes.

Once there is suspicion of an AIH diagnosis in a patient with HIV, they should be treated in a similar manner to immune competent patients. The first line includes prednisone or prednisolone (0.5-1 mg/kg per day), and azathioprine (1-2 mg/kg per day), gradually decreasing steroid doses and continuing maintenance treatment based on azathioprine. It is essential to continue antiretroviral therapy[19].

As in this case, in all reported AIH and HIV cases in literature, the development of hepatitis generated a suspicion of toxicity caused by antiretroviral therapy. In the recent AIH guide[19], a protocol was proposed in order to differentiate it from DILI (Figure 3). Typically, the antiretroviral therapy has a pattern of hepatocellular or cytolytic injury[13,14,22]. The most common histologic findings in hepatotoxicity related to drugs are microvesicular steatosis, acute hepatitis, eosinophilic infiltration and cholestatic injury[23]. DILI associated with antiretroviral therapy is a major problem; some of the nucleoside reverse transcriptase inhibitors (zidovudine, didanosine, stavudine, abacavir) are associated with mitochondrial toxicity and hepatic steatosis. The non-nucleoside inhibitors, such as Efavirenz and Nevirapine are associated with an increase of liver enzymes, and the latter to hypersensitivity reactions. Protease inhibitors (Indinavir, Saquinavir, Nelfinavir, Ritonavir, Lopinavir-

ritonavir, Fosamprenavir, Atazanavir and Tipranavir) are associated with an increase of transaminases, and some, such as Indinavir and Atazanavir to indirect hyperbilirubinemia[24].

Upon suspicion of DILI, suspicious medication should be removed and the liver profile monitored until recovery, later each potentially involved medication reapplied. There are frequent difficulties in identifying the medications that are responsible, since several suspects can coexist, as happens with antituberculosis drugs (Rifampin, Isoniazid, Pyrazinamide), antiretroviral drugs (Efavirenz, Nevirapine, protease inhibitors) and other therapies such as Trimetoprim-sulfamethoxazole. If necessary, an attempt should be made to reintroduce the medications, avoiding the more suspicious agent, as there is a possibility of DILI with greater severity[25].

This is a case in point that does not fully represent the behavior of the autoimmune hepatitis in HIV patients. Therefore, more studies are needed in this population in order to achieve a better understanding of these illnesses.

In conclusion, autoimmune diseases should be taken into consideration in patients with HIV infection, especially those who receive cART therapy, as the immunological recovery can be unleashing them. Likewise, for patients with HIV who show elevated aminotransferase levels, autoimmune hepatitis should be taken into account as one of the possible causes and perform a diagnostic approach to differentiate it from other etiologies. This allows the appropriate treatment and avoids the prolonged suspension of antiretrovirals, and the complications arising from poor virologic control.

ARTICLE HIGHLIGHTS

Case characteristics

A 26-year-old male, diagnosed with human immunodeficiency virus (HIV) infection and treated with Efavirenz/Tenofovir disoproxil fumarate/Emtricitabine, was admitted to the emergency room due to jaundice, anti-retroviral treatment was suspended.

Clinical diagnosis

Jaundice without right upper quadrant pain or hepatomegaly.

Differential diagnosis

Drug-induced liver injury, viral hepatitis, alcoholic liver disease, neoplasm, acquired immune deficiency syndrome cholangiopathy.

Laboratory diagnosis

Hyperbilirubinemia with a predominance of direct bilirubin, severe elevation of transaminases and prolongation of the prothrombin time. The serology for hepatotropic viruses was negative (A, B, C and E), viral loads for virus B viral hepatitis, C viral hepatitis, Epstein Bar and cytomegalovirus were undetectable. The antinuclear antibodies were positive with mottled pattern, negative anti-mitochondrial and anti-muscle antibodies and high levels of immunoglobulin G.

Imaging diagnosis

The hepato-biliary ultrasound and portal doppler were normal.

Pathological diagnosis

Lymphoplasmacytic inflammatory infiltration with eosinophils and severe interface activity, hepatocytes with peri-central inflammation and focal necrosis ("compatible with autoimmune hepatitis").

Treatment

Prednisolone of 1 mg/kg per day following tapered doses, Azathioprine 100 mg/qd and cART (Tenofovir disoproxil fumarate/Emtricitabine and Raltegravir).

Related reports

After literature search, nine reports with only 22 cases of patients with both entities were found, none of them described in Latin America.

Term explanation

Autoimmune hepatitis is a chronic inflammation of the liver of unknown cause, pathogenesis includes environmental triggers, failure of immune tolerance mechanisms, and a genetic predisposition that induce a T cell-mediated immune attack characterized with continuing hepatocellular necroinflammatory and fibrotic process. The diagnosis is based on histologic abnormalities, clinical and laboratory findings, abnormal levels of immunoglobulin G, and one or more characteristic autoantibodies.

Experiences and lessons

Autoimmunity in patients with HIV infection on cART is uncommon, nevertheless in some clinical scenarios should be considered. The differentiation among autoimmune hepatitis (AIH), drug induced liver injury or infectious hepatitis can be challenging and needs an extensive work-up.

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