ISSN 2220-3176 (online)

# World Journal of *Clinical Infectious Diseases*

*World J Clin Infect Dis* 2017 May 25; 7(2): 11-37

Published by Baishideng Publishing Group Inc

WJCID

World Journal of Clinical Infectious Diseases

A peer-reviewed, online, open-access journal of clinical infectious diseases

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2016-2019

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World Journal of Clinical Infectious Diseases

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Quarterly Volume 7 Number 2 May 25, 2017

*World Journal of Clinical Infectious Diseases*
Volume 7 Number 2 May 25, 2017

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NAME OF JOURNAL
*World Journal of Clinical Infectious Diseases*

ISSN
ISSN 2220-3176 (online)

LAUNCH DATE
December 30, 2011

FREQUENCY
Quarterly



EDITORIAL OFFICE
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*World Journal of Clinical Infectious Diseases*
Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
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PUBLISHER
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PUBLICATION DATE
May 25, 2017



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ONLINE SUBMISSION
http://www.f6publishing.com

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*World J Clin Infect Dis* 2017 May 25; 7(2): 11-20

DOI: 10.5495/wjcid.v7.i2.11

ISSN 2220-3176 (online)

REVIEW

# Management of periprosthetic infections

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

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Author contributions: Vilchez-Cavazos F and Villarreal-Villarreal G wrote the paper; Peña-Martinez V and Acosta-Olivo C collected data and literature.

Conflict-of-interest statement: Authors declare no conflict of interests for this article.



Manuscript source: Invited manuscript

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Received: August 27, 2016
Peer-review started: August 29, 2016
First decision: November 21, 2016
Revised: December 6, 2016
Accepted: January 20, 2017
Article in press: January 22, 2017
Published online: May 25, 2017

## 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.

Vilchez-Cavazos F, Villarreal-Villarreal G, Peña-Martinez V, Acosta-Olivo C. Management of periprosthetic infections. *World J Clin Infect Dis* 2017; 7(2): 11-20 Available from: URL: http://www.wjgnet.com/2220-3176/full/v7/i2/11.htm DOI: http://dx.doi.org/10.5495/wjcid.v7.i2.11

## 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

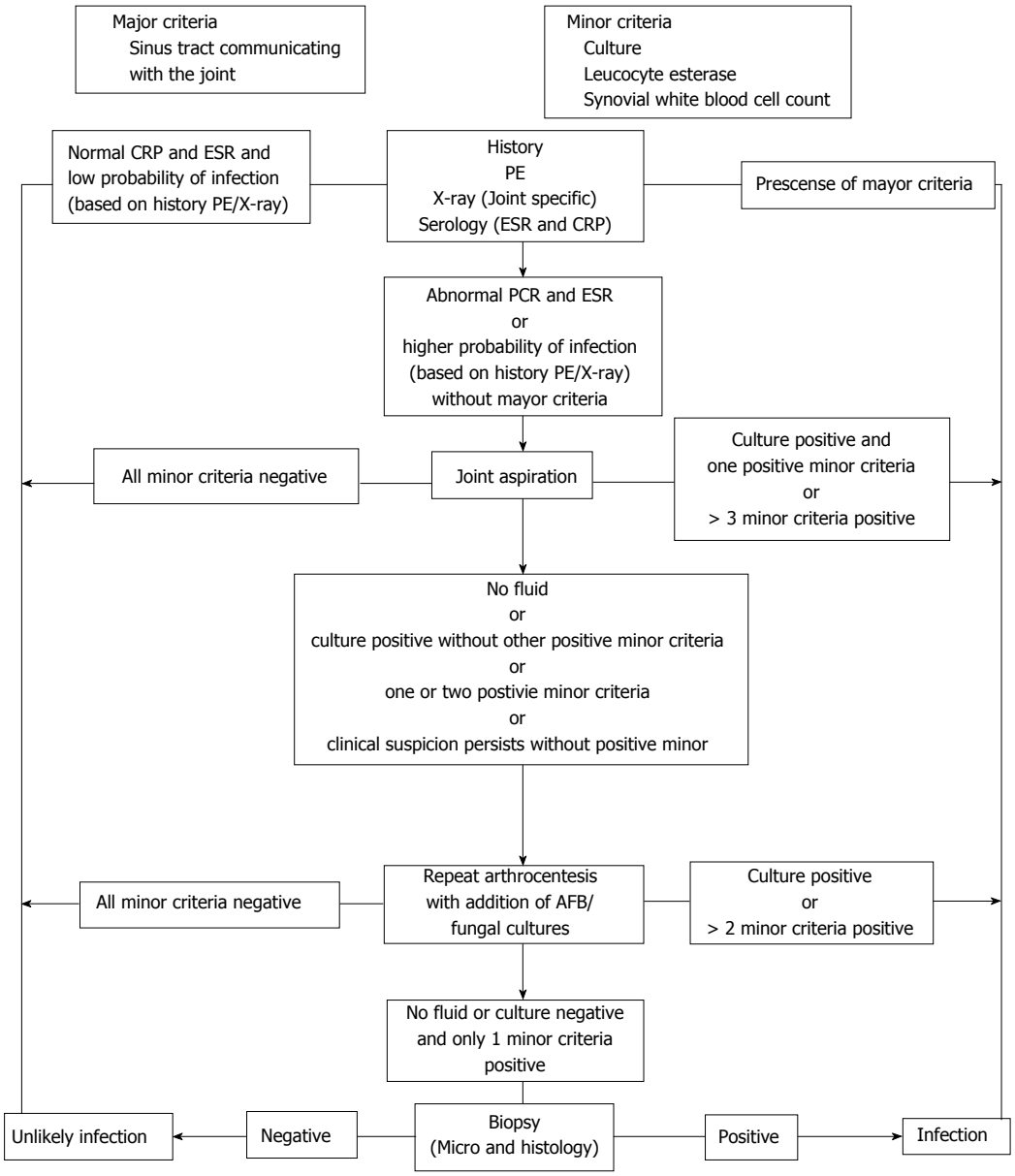


**Figure 1 Simplified algorithm for approaching a patient with a probable periprosthesic joint infection, proposed by Della Valle *et al*[12].** CPR: C-reactive protein; ESR: Erythrocyte sedimentation rate; PE: Physical examination.

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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**P- Reviewer**: Anand A, Cui Q, Drosos GI **S- Editor**: Ji FF
**L- Editor**: A **E- Editor**: Li D

World Journal of Clinical Infectious Diseases

Submit a Manuscript: http://www.f6publishing.com

*World J Clin Infect Dis* 2017 May 25; 7(2): 21-31

DOI: 10.5495/wjcid.v7.i2.21

ISSN 2220-3176 (online)

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.

Supported by WOS-A grant from Department of Science and Technology, New Delhi, Government of India to Iyer LR, No. SR/WOS-A/LS-98/2007; "Programme support on molecular parasitology", Department of Biotechnology, New Delhi, India to Paul J, No. BT/01/CEIB/11/v/08.

Institutional review board statement: All stool samples from patients were taken after informed consent and ethical permission was obtained for participation in the study (Government of India, Institute Ethics committee, Safdarjung hospital and VMMC, letter no VMMC/SJH/PROJECT/JAN-14/21).

Conflict-of-interest statement: To the best of our knowledge, no conflict of interest exists.

Data sharing statement: None.



Manuscript source: Invited manuscript

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Received: September 23, 2016
Peer-review started: September 26, 2016
First decision: November 2, 2016
Revised: January 11, 2017
Accepted: February 10, 2017
Article in press: February 13, 2017
Published online: May 25, 2017

## 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)<br>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

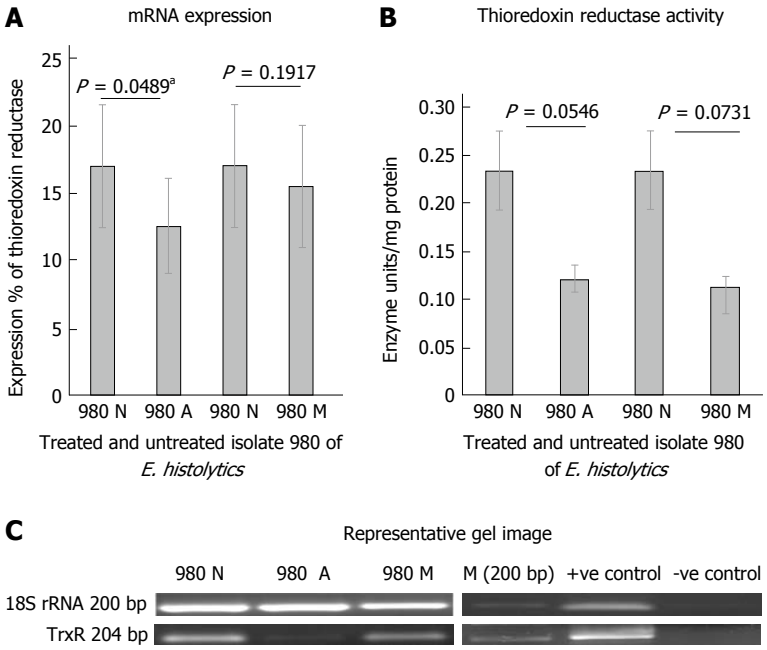


**Figure 1 mRNA expression and activity levels of thioredoxin reductase in clinical isolate 980 of *Entamoeba histolytica* during treatment with antiamoebic drugs.** A: Graphical representation of densitometric data from semiquantitative RT-PCR analysis of Thioredoxin reductase in clinical isolate 980. 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

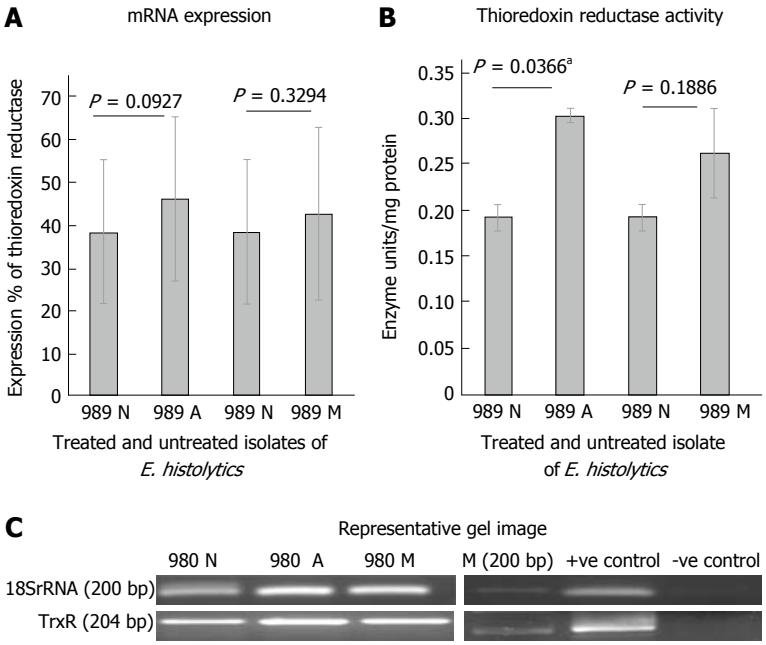


**Figure 2 mRNA expression and activity levels of thioredoxin reductase in clinical isolates 989 of *Entamoeba histolytica* during treatment with antiamoebic drugs.** A: Graphical representation of densitometric data from semiquantitative RT-PCR analysis of Thioredoxin reductase in clinical isolate 989, 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

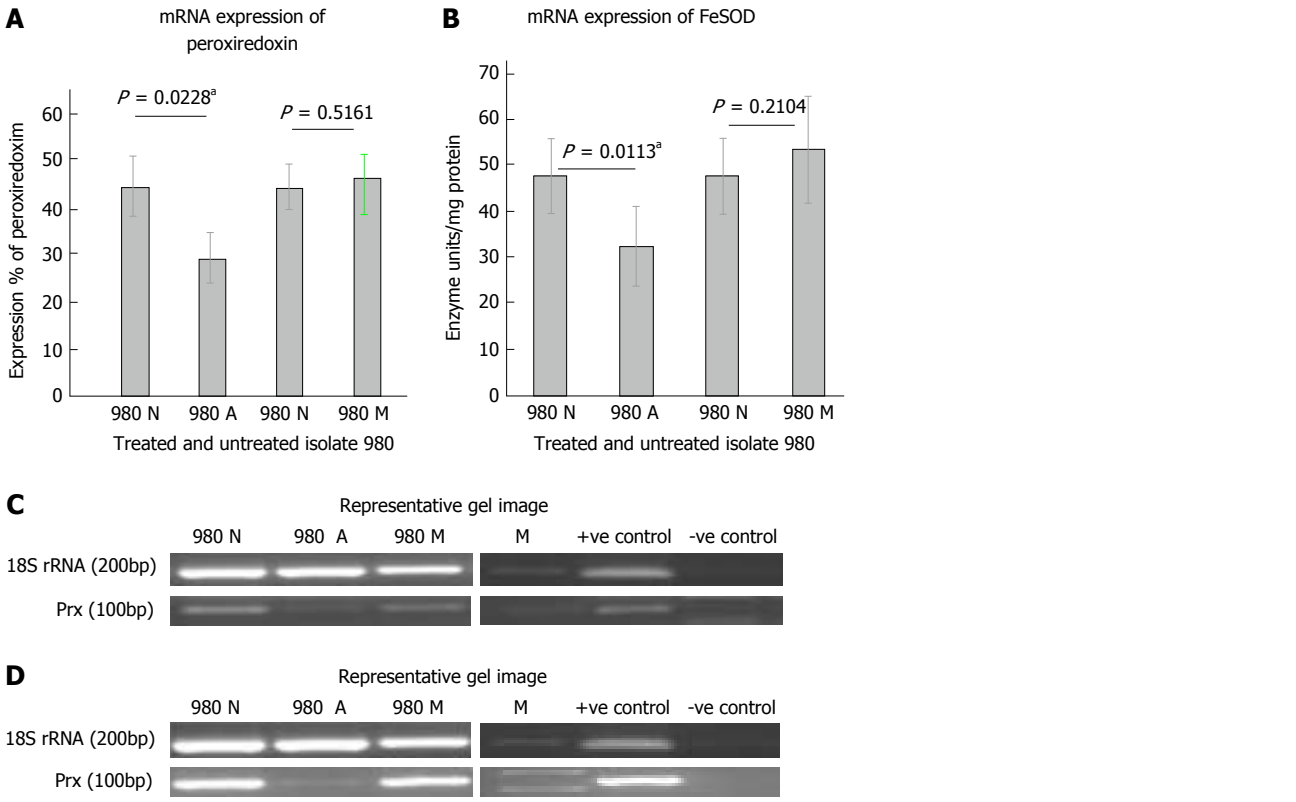


**Figure 3 mRNA expression and activity levels of peroxiredoxin and FeSOD in clinical isolate 980 of *Entamoeba histolytica* during tatment with antiamoebic drugs.** A: Graphical representation of densitometric data from semiquantitative RT-PCR analysis of Peroxiredoxin in clinical isolate 980, untreated (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.

## ACKNOWLEDGMENTS

The authors gratefully acknowledge Dr. Mukul Singh and Dr. Amit Dubey of Department of Pathology, Safdarjung Hospital, New Delhi for kindly providing the clinical samples and Professor Sudha Bhattacharya of School of Environmental Sciences, Jawaharlal Nehru University for the use of Entamoeba culturing facilities and her valuable inputs to the project.

## 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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**P- Reviewer**: García-Elorriaga G, Sugawara I **S- Editor**: Ji FF
**L- Editor**: A **E- Editor**: Li D

World Journal of Clinical Infectious Diseases

Submit a Manuscript: http://www.f6publishing.com

*World J Clin Infect Dis* 2017 May 25; 7(2): 32-37

DOI: 10.5495/wjcid.v7.i2.32

ISSN 2220-3176 (online)

CASE REPORT

# Disseminated cryptosporidiosis: Case report and literature review

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Supported by Council of Scientific and Industrial Research, Government of India.

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.

Conflict-of-interest statement: Authors declare no conflict of interest.



Manuscript source: Invited manuscript

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Received: April 18, 2016
Peer-review started: April 19, 2016
First decision: May 17, 2016
Revised: January 31, 2017
Accepted: February 20, 2017
Article in press: February 21, 2017
Published online: May 25, 2017

## 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.

Khalil S, Mirdha BR, Paul J, Panda A, Singh Y. Disseminated cryptosporidiosis: Case report and literature review. *World J Clin Infect Dis* 2017; 7(2): 32-37 Available from: URL: http://www.wjgnet.com/2220-3176/full/v7/i2/32.htm DOI: http://dx.doi.org/10.5495/wjcid.v7.i2.32

## 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.

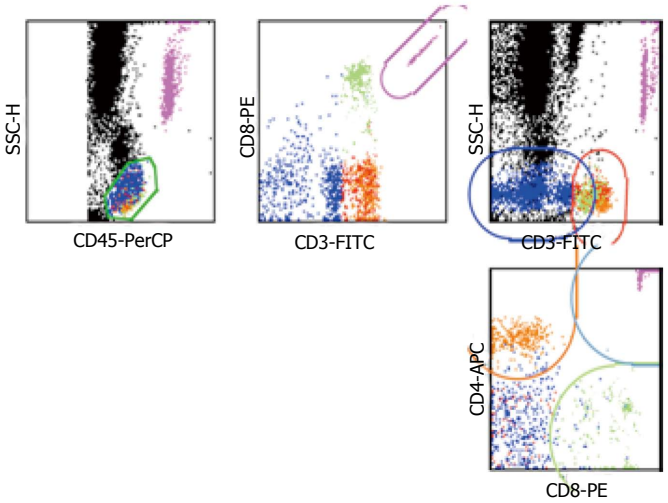
## CASE REPORT

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 ($5.25 \times 10^5$/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.**

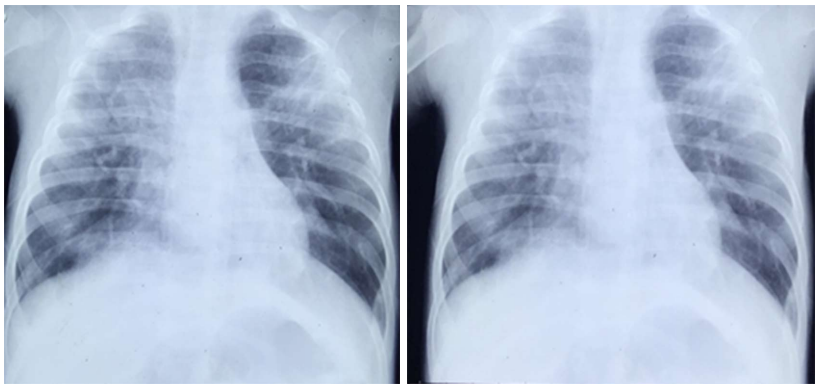


**Figure 2 Chest X-ray of CD8+ deficient child showing bilateral infiltrates.**

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′<br>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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**P- Reviewer**: Gonzalez-Granado LI, Moschovi MA, Nagata T, Pourshafie MR
**S- Editor**: Ji FF **L- Editor**: A **E- Editor**: Li D

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