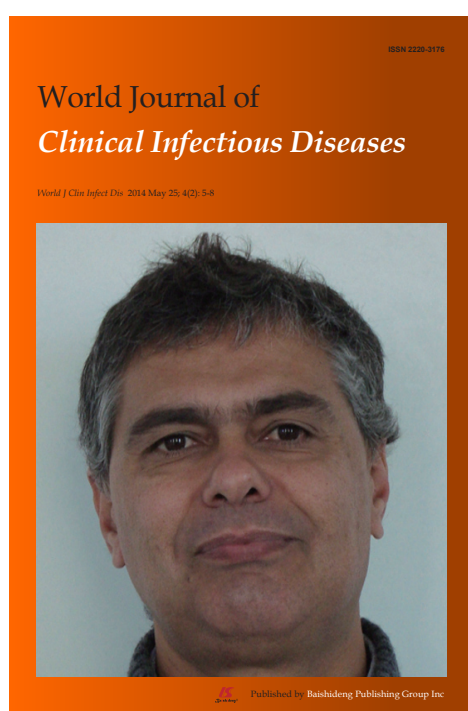
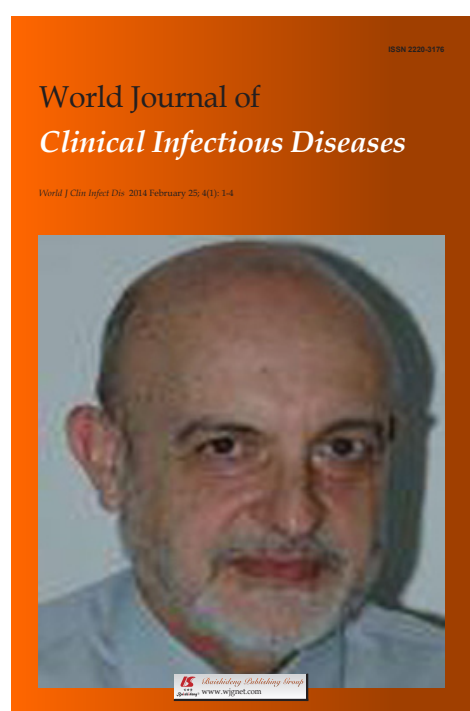


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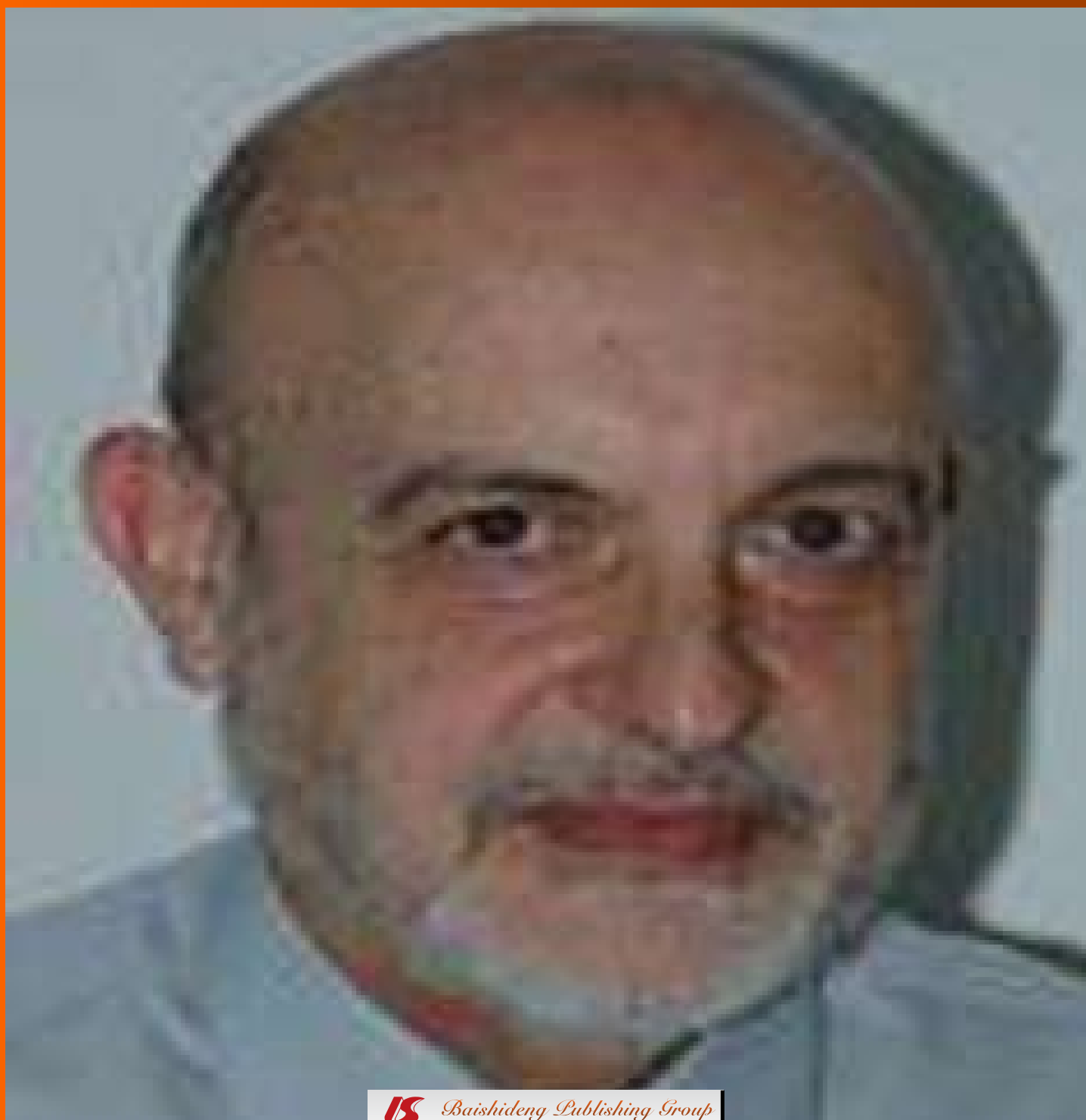
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# World Journal of *Clinical Infectious Diseases*

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Sub-acute endocarditis by *Corynebacterium straitum*: An often ignored pathogen

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## Sub-acute endocarditis by *Corynebacterium straitum*: An often ignored pathogen

Vratika Agarwal, Valay Parikh, Mayur Lakhani, Chitradeep De, Apurva Motivala, Neville Mobarakai

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gens in invasive disease, mortality and morbidity can be prevented with initiation of early appropriate therapy.

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**Key words:** *Corynebacterium straitum*; Endocarditis; Commensals; Catheter associated infections; Iatrogenic

**Core tip:** This report highlights the pathogenicity of previously considered commensals like *Corynebacterium straitum* in severe human disease. Clinicians and microbiologists should not overlook the potential virulence of commensals in appropriate clinical situations. Furthermore, our case highlights an important point regarding the emergence of new pathogens in the etiology of infective endocarditis and also throws light on the increased incidence of endocarditis with the use of indwelling catheters and aggressive invasive medical management. As this issue is of growing concern with respect to infections that are easily avoidable.

### Abstract

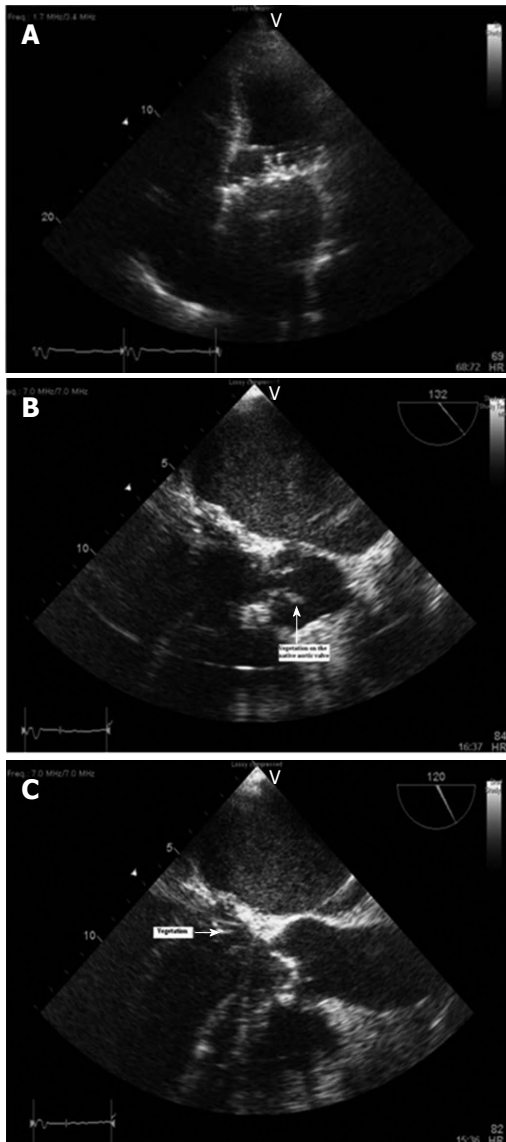
With the emergence of novel etiologic organisms, pan-resistance, and invasive medical care infective endocarditis continues to be evasive, requiring newer approaches and modified treatment guidelines. Presented here is the case of a 75-year-old male with history of systolic heart failure with an automatic internal cardioverter defibrillator (AICD) implantation and a prosthetic mitral valve who presented with generalized malaise and progressive shortness of breath for 6 d. He was found to have positive blood cultures for gram positive rod shaped bacteria identified as *Corynebacterium straitum*, but was not considered as the etiological pathogen initially as it a usual skin contaminant. Later this bacterium was found to be the causative agent for the patient's endocarditis. This case highlights the importance of identifying the role of this uncommon commensal in invasive disease. With the use of effective antibiotic regimen and awareness of these new patho-

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### INTRODUCTION

The epidemiological profile of infective endocarditis has changed drastically over the last several years with an estimated 10000 to 15000 new cases of IE diagnosed each year in United States. The number of cases of hospital acquired endocarditis has been on a rise with an estimated 7.5% to 29% of total cases of endocarditis in tertiary-care centre<sup>[1]</sup>. Intravenous indwelling catheters being one of the important causes of amplified risk of bacteremia.





**Figure 1** Transesophageal echocardiogram. A: Transthoracic echocardiographic image showing apical 4-chambered view showing possible vegetation on prosthetic mitral valve; B: Transesophageal echocardiogram showing sub-centimeter vegetation on the aortic valve; C: Transesophageal echocardiogram showing mobile vegetation at the prosthetic mitral valve annulus.

*Corynebacterium straitum* (CS) previously considered a saprophyte on skin and mucosa is now emerging as a causative organism for various infections, not only in immunocompromised but in immunocompetent individuals as well. Positive blood cultures for CS should not be neglected. The exact infectious potential of these bacteria and their judicious antimicrobial treatment is a challenging but necessary task.

## CASE REPORT

A 75-year-old male with history of systolic heart failure with an automatic internal cardioverter defibrillator implantation and a prosthetic mitral valve presented with generalized malaise and progressive shortness of breath for 6 d. Patient also noticed worsening of lower

extremity edema. He denied chest pain, fever, cough and palpitations. Physical exam revealed a new systolic murmur, bilateral crackles and positive pitting pedal edema. Based on initial assessment a diagnosis of acute decompensated heart failure secondary to suspected prosthetic valve infective endocarditis was made. Over the next 48 h four sets of blood cultures were drawn. Transthoracic echocardiography showed dilated left atrium and possible mitral valve vegetation (Figure 1A). In addition to the symptomatic treatment of heart failure, daptomycin, gentamicin, ceftriaxone and rifampin were started empirically. Transesophageal echocardiography (TEE) revealed a subcentimeter vegetation on the aortic valve (Figure 1B) in addition to small, protruding, echogenic, mobile vegetation at the prosthetic mitral valve annulus (Figure 1C). Four sets of blood cultures drawn 24 h apart, all grew CS. Initially, was thought to be a contaminant but later confirmed as a pathogen with gram stain and microbiological characterization which included acid fast staining, chemical and antibiotic testing. Antimicrobial susceptibility of the above organism by clinical and laboratory standards institute showed sensitivity of the organism to vancomycin, ampicillin (intermediate sensitivity), daptomycin, rifampin and gentamicin. Based on antibiotic sensitivity, daptomycin was discontinued and ampicillin was added. Ceftriaxone was continued initially as the patient also has a urinary tract infection but was later discontinued after second set of cultures showed the same results and urine cultures were negative. No other bacteria were isolated from the blood cultures. Subsequent blood cultures drawn 4 d later were negative. Patient's condition improved over a period of 3 wk. Antibiotics were continued for a total of 6 wk.

One month prior to this admission, patient was admitted to a tertiary care center for diverticular bleed, which was treated with IV resuscitation via an internal jugular venous access. This was the presumed source of infection.

## Microbiology

Nondiphtherial corynebacteria also known as coryneforms, are a widely diverse collection of bacteria that are grouped together on basis of their 16S rDNA<sup>[2]</sup>. The diversity of the group is further exemplified by the wide range of guanine-plus-cytosine content of the nucleotides. Although frequently considered as colonizers or contaminants they have been associated with invasive disease, particularly in immunocompromised patients. These organisms have been specifically implicated in bacteremia associated with catheterizations, shunts, pacemakers causing meningitis, osteomyelitis, prosthetic heart valve endocarditis and peritonitis, often seen in patients undergoing dialysis or have associated empyema, pneumonia and skin infections<sup>[3,4]</sup>. Patients infected with nondiphtherial organisms usually have significant medical co-morbidity or immunosuppression.

CS is one of the less known nondiphtherial species belonging to the genus corynebacteria. Frequently

distinguished from other coryneform bacteria by being non acid fast, nitrate reducing, utilizing glucose and sucrose and having colonies that are white grey or yellowish green in color. This species is non-motile. Microscopically they are larger when compared to the other coryneform bacteria and on gram stain have a typical striated appearance. Therefore named *Corynebacterium striatum*. CS is found in the anterior nares and on the skin, face and upper torso of normal individuals. There is a growing concern for its role as a pathogen in various disease entities, as noted in few case studies<sup>[5-7]</sup>.

## DISCUSSION

There has been a steep rise in the number of reported cases of health care acquired infective endocarditis over the last decade. This is most likely due to the vast use of invasive procedures in people with or without pre-existing valvular disease<sup>[8]</sup>. Effective antibiotic regimen has managed to curb the traditional etiological organisms, but there is growing concern for identification of newer unrecognized pathogens. CS generally considered a commensal, has now been implicated as a causative organism in various infections. Although nontoxigenic, this organism has been associated with invasive infections in patients with implanted cardiac device and prosthetic valves<sup>[9]</sup>, usually in an immunocompromised setting. There have been reports of CS bacteremia attributed to indwelling venous catheters<sup>[10]</sup>. CS infection along with *C. jeikeium* has been shown to be associated with nosocomial risk factors in some studies<sup>[11]</sup>. Most of the reported CS isolates have been susceptible to a wide spectrum of antibiotics. There have been few reported cases of multi drug resistant CS<sup>[12]</sup>. In general they are resistant to penicillin's but have been reported to be susceptible to B-lactams and vancomycin. There are no established guidelines for the management of corynebacteria yet. But in the light of impending multidrug resistance and nosocomial infections, there is need for appropriate guidelines to be established. Positive blood cultures for *Corynebacterium striatum* should not be neglected as a contaminant because of lack of data involving its role as a pathogen, especially in a hospital environment. Recognition and classification of diphtherial organisms continues to be a challenge for various laboratories as there is discrepancy in diagnosis of contamination in opposition to causative pathogen<sup>[13]</sup>. Delay in treatment increases morbidity and mortality.

This report highlights the pathogenicity of previously considered commensals like *Corynebacterium striatum* in severe human disease. Clinicians and microbiologists should not overlook the potential virulence of commensals in appropriate clinical situations.

## COMMENTS

### Case characteristics

Acute decompensated heart failure with fluid overload. Found to be febrile with

positive blood cultures.

### Clinical diagnosis

Sub-acute endocarditis due to *Corynebacterium striatum* (CS).

### Differential diagnosis

Most Coryneform bacteria are commensals on human skin and are ubiquitous in our environment. Initially positive blood cultures for this organism was thought to be a contaminant, but was later found to be the etiological agent of endocarditis.

### Laboratory diagnosis

Organisms isolated from blood culture underwent staining with Gram's method and acid fast staining. Microbiological characterization was also performed.

### Imaging diagnosis

Presence of vegetation was identified using transthoracic and transesophageal echocardiography.

### Pathological diagnosis

Sub-acute endocarditis due to invasive CS infection.

### Treatment

Antibiotic therapy with Rifampin, ampicillin, and gentamycin. There is lack of data with regards to treatment of endocarditis due to CS.

### Term explanation

Cardioverter defibrillator: An implantable cardioverter defibrillator is a small device that's placed in the chest or abdomen. Used to treat arrhythmias; Vegetation: Endocarditis is characterized by a prototypic lesion, the vegetation, which is a mass of platelets, fibrin, microcolonies of microorganisms, and scant inflammatory cells.

### Experiences and lessons

Clinicians and microbiologists should not overlook the potential virulence of commensals like CS in appropriate clinical situations.

### Peer review

This is just a single case report describing a human endocarditis caused by CS. The case report is interesting and the manuscript is well written.

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- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

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#### Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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# World Journal of *Clinical Infectious Diseases*

*World J Clin Infect Dis* 2014 May 25; 4(2): 5-8





**CASE REPORT**

5

Unsuspected imported malaria in a case of sudden infant death

*Pusiol T, Lavezzi AM, Radice F, Alfonsi G, Maturri L*

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*World Journal of Clinical Infectious Diseases*  
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## Unsuspected imported malaria in a case of sudden infant death

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### Abstract

Here we describe the case of a 4-mo-old female who died suddenly without any apparent cause that was initially mistaken as a case of sudden infant death syndrome. Histologic observation of brain sections revealed blue-black bodies in erythrocytes of the blood vessels, suggestive of specific stages of the hematic schizogonic cycle. Further examinations revealed hemozoin and hemosiderin deposits in the parenchyma of all organs, leading to the diagnosis of malaria by *Plasmodium falciparum* (*P. falciparum*). The death occurred in Italy, the native country of the infant, two weeks after a Christmas holiday spent in Pakistan, the parents' birthplace, which has a high malarial endemicity. As this case demonstrates, the diagnosis of malaria should always be considered as a differential diagnosis in subjects, including infants, that die unexpectedly after returning from *P. falciparum* endemic areas.

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**Key words:** Parasitemia; *Plasmodium falciparum*; Protozoa; Sudden infant death; Unsuspected imported malaria

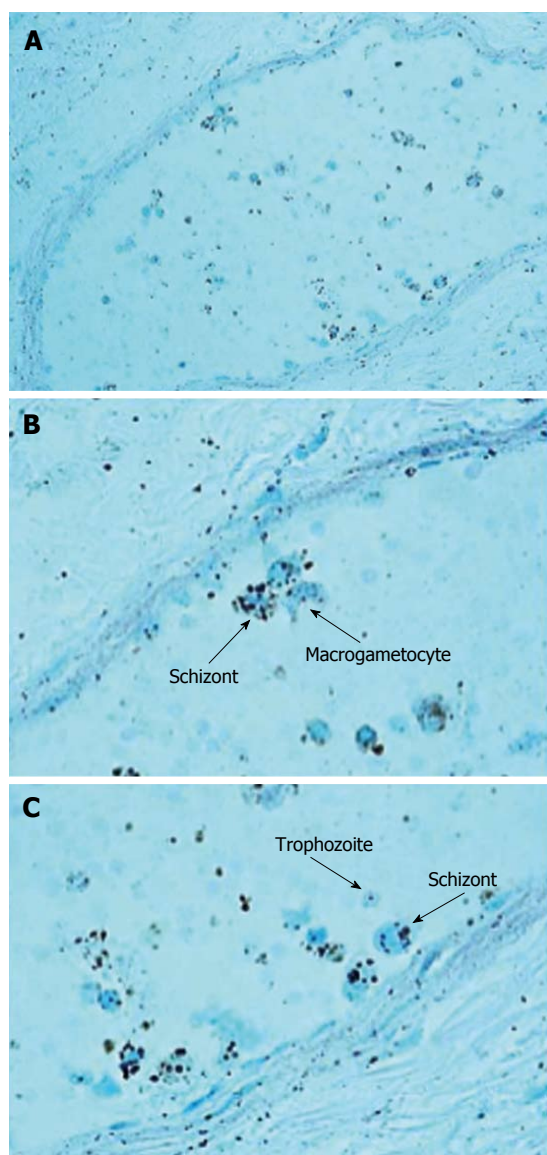
**Core tip:** This report describes the case of a 4-mo-old baby whose sudden death was initially attributed to sudden infant death syndrome. Histologic examination of organ specimens unexpectedly revealed blue-black bodies in erythrocytes, suggestive of specific stages of the hematic schizogonic cycle, and hemozoin and hemosiderin deposits in the parenchyma of all organs. These observations led to the diagnosis of death from malaria by *Plasmodium falciparum*. In support of this diagnosis, the baby had recently returned from a stay in Pakistan, a region with high malarial endemicity.

Pusiol T, Lavezzi AM, Radice F, Alfonsi G, Maturri L. Unsuspected imported malaria in a case of sudden infant death. *World J Clin Infect Dis* 2014; 4(2): 5-8 Available from: URL: <http://www.wjgnet.com/2220-3176/full/v4/i2/5.htm> DOI: <http://dx.doi.org/10.5495/wjcid.v4.i2.5>

### INTRODUCTION

Malaria is an infection caused by the protozoa *Plasmodium* with high morbidity and mortality in endemic areas, including Asia and Africa<sup>[1-3]</sup>. The virulence of the malarial agents is a consequence of a number of features, the most important of which is the tendency for parasitized erythrocytes, with the consequent occlusion of the capillaries and blockade of circulation<sup>[4,5]</sup>. The clinical syndromes associated with *Plasmodium* infections range from asymptomatic parasitemia to high fever, chills, convulsions, coma and death<sup>[6,7]</sup>. In infants, in particular, the typical signs of malaria (*e.g.*, febrile illness), are generally absent, and include only sudden behavioral changes like irritability, lethargy, drowsiness<sup>[1,6]</sup>. Thus, infants are at increased risk for a rapid disease progression due to the undiagnosed infection. In the absence of a timely diagnosis, erythrocyte parasitemia may reach critical values





**Figure 1** Different stages of the hematic schizogonic cycle of malarial parasite (schizont, trophozoite and crescent-shape macrogametocyte) in a splenic vessel. Giemsa stain, magnification A: 20 ×; B: 100 ×; C: 100 ×.

and cause massive hemolysis and multiple organ dysfunction, resulting in death. The World Health Organization estimates that in malaria-endemic areas, infants become vulnerable to *Plasmodium* at around three months of age, when immunity acquired from the mother starts to wane<sup>[2]</sup>. Here, we report a case of an unsuspected and postponed malaria diagnosis in a 4-mo-old female, who died suddenly in Italy, her native country, two weeks after a Christmas holiday spent in her parent's birthplace, Pakistan, which has high malarial endemicity.

## CASE REPORT

The case of a 4-mo-old female who died suddenly during sleep without any apparent cause was sent as a suspected case of sudden infant death syndrome to the "Lino Rossi" Research Center of the Milan University, according

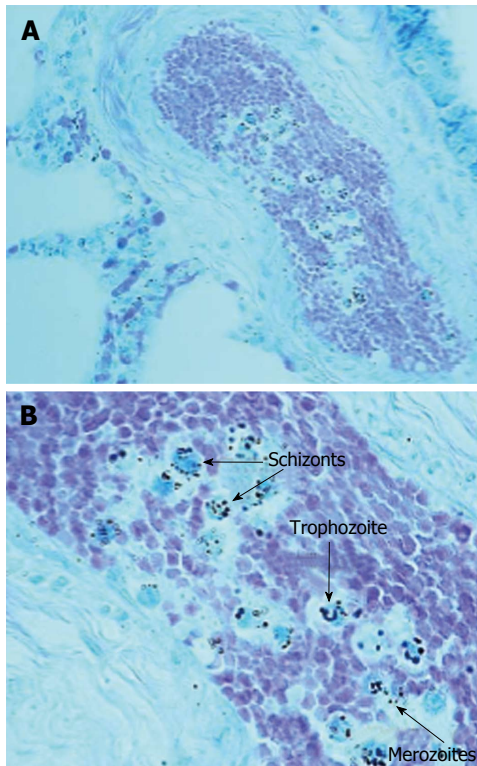
to Italian law: 31/2006 "Regulations for Diagnostic Post Mortem Investigation in Victims of the Sudden Infant Death Syndrome (SIDS) and Unexpected Fetal Death".

The parents brought their daughter to northern Pakistan, their region of origin, in occasion of the Christmas holiday for around 50 d (from November 20, 2013 to January 10, 2014). During this stay, the baby was in good health. Approximately 15 d before the end of the visit, the parents noticed signs of a mosquito bite on the baby's face. On the tenth days after their return to Italy, although showing no signs of fever, the baby did not eat and showed a lack of responsiveness. For this reason, the parents brought her directly to the nearest hospital, where she arrived with no signs of heartbeat or breathing. Despite the attempts of resuscitation, physicians confirmed the absence of vital signs and death.

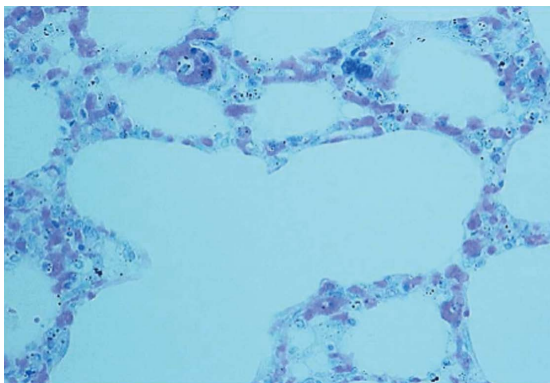
The autopsy examination did not show a clear cause of death, and excluded important disease processes and/or congenital malformations. An in-depth study of the autonomic nervous system, performed according to the above-mentioned Italian law in case of sudden infant death, did not detect any alteration, particularly of the brainstem vital centers. However, examination of hematoxylin/eosin-stained brain sections highlighted the presence of small blue-black bodies within erythrocytes in capillaries, indicative of infection from malarial parasites. The examination was then extended to samples of all organs. Histologic sections were processed with Giemsa staining to determine the intensity and distribution of the parasite in the different stages of the hematic schizogonic cycle in the capillaries of each organ. It was possible to recognize trophozoites, schizonts, merozoites and crescent-shape macrogametocytes, which are a distorted form of gametocyte specific to *Plasmodium falciparum* that allow differentiation from other types of malarial infection (Figures 1-3). The Perls method for iron was also used to distinguish the intra- and extra-erythrocyte hemozoin and hemosiderin, the malaria pigments arising from rupture of mature schizonts (Figures 4 and 5). Pigmented phagocytic cells were frequently found dispersed in all organs. The final diagnosis was imported acute malignant malaria from *Plasmodium falciparum*.

## DISCUSSION

Malaria disease begins with the injection of sporozoites from an infected female *Anopheles* mosquito into the skin of a human host. The sporozoites primarily reach the liver and then develop within the hepatocytes through schizogonic divisions. This leads to the formation of numerous merozoites that, immediately after release in the bloodstream, parasitize red blood cells, thus initiating the intra-erythrocytic cycle, which is responsible for the initiation of clinical malaria<sup>[8,9]</sup>. *Plasmodium* parasites therefore have two obligatory intracellular development phases, first in hepatocytes and subsequently in erythrocytes. We believe that in this case, the severe congestion of parasitized erythrocytes observed in microvessels of



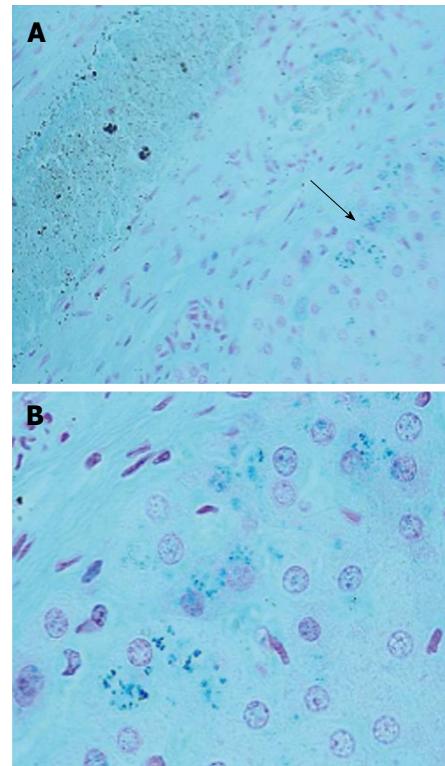
**Figure 2** Malarial parasites in a pulmonary vessel. Giemsa stain showing merozoite, schizont and trophozoite stages; magnification A: 20 ×; B: 40 ×.



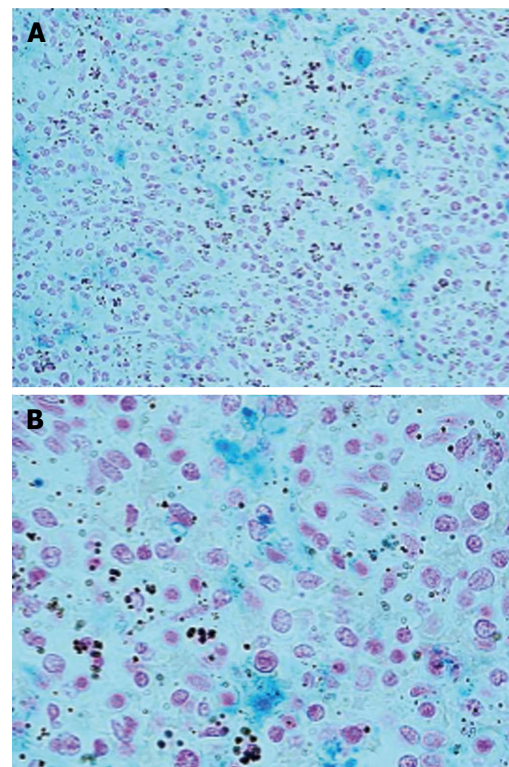
**Figure 3** Numerous schizonts in the capillaries of pulmonary alveolar septa. Giemsa stain, 20 × magnification.

all organs, and especially in the brain, played a crucial role in the pathogenic mechanism of the sudden death.

The baby returned from a trip in Pakistan, a region where malaria continues to be a serious public health problem. Despite a well-established malaria control program, 500000 malaria infections and 50000 malaria-attributable deaths occur each year in Pakistan<sup>[10,11]</sup>. Although polymerase chain reaction has been introduced to detect *Plasmodium*-positive samples, the Giemsa staining method remains, for simplicity and low cost, the gold standard for the diagnosis of *Plasmodium* infections<sup>[12-14]</sup>. This report highlights that a diagnosis of malaria must be considered as an important differential diagnosis in subjects who have recently stayed in malarial endemic regions, with or without specific clinical symptoms. Even if the



**Figure 4** Malarial pigment grain accumulation in the splenic reticuloendothelial system. Perls stain showing hemozoin and hemosiderin; magnification A: 40 ×; B: 100 ×.



**Figure 5** Malarial pigment grain accumulation in the splenic reticuloendothelial system. Perls staining method for iron showing hemozoin and hemosiderin; magnification A: 20 ×; B: 100 ×.

malaria is an infrequently encountered infection in non-



endemic areas, particularly in Europe<sup>[15]</sup>, a high degree of suspicion is needed. Furthermore, proper questioning by a doctor is fundamental in the diagnosis of imported malaria, especially when the clinical signs are non-specific and sometimes misleading. This should be applied also in cases of infants who die suddenly in the first months of life, which often occur during sleep and are classified as SIDS.

## COMMENTS

### Case characteristics

The paper describes a case of a 4-mo-old female who died suddenly without any apparent cause, which was initially mistaken as a sudden infant death syndrome (SIDS) case.

### Clinical diagnosis

Clinical diagnosis was SIDS and other death cause were not considered.

### Pathological diagnosis

Acute malignant malaria from *Plasmodium falciparum*.

### Treatment

Despite the attempts of resuscitation, physicians established the non-resumption of vital signs and death.

### Term explanation

SIDS is defined as the sudden death of an infant under one year of age that remains unexplained after a thorough case investigation, including performance of a complete autopsy, examination of the death scene, and a review of the clinical history.

### Experiences and lessons

This case with a post-mortem diagnosis of malaria is important from a medico-legal point of view because of the potential responsibility of the physician treating a patient of any age who has returned from endemic areas.

### Peer review

The manuscript is a case report of interest in the area of health, as it can lead to greater awareness among those responsible for the area, to the attention of individuals and newborns who travel from endemic areas in malaria to non-endemic areas.

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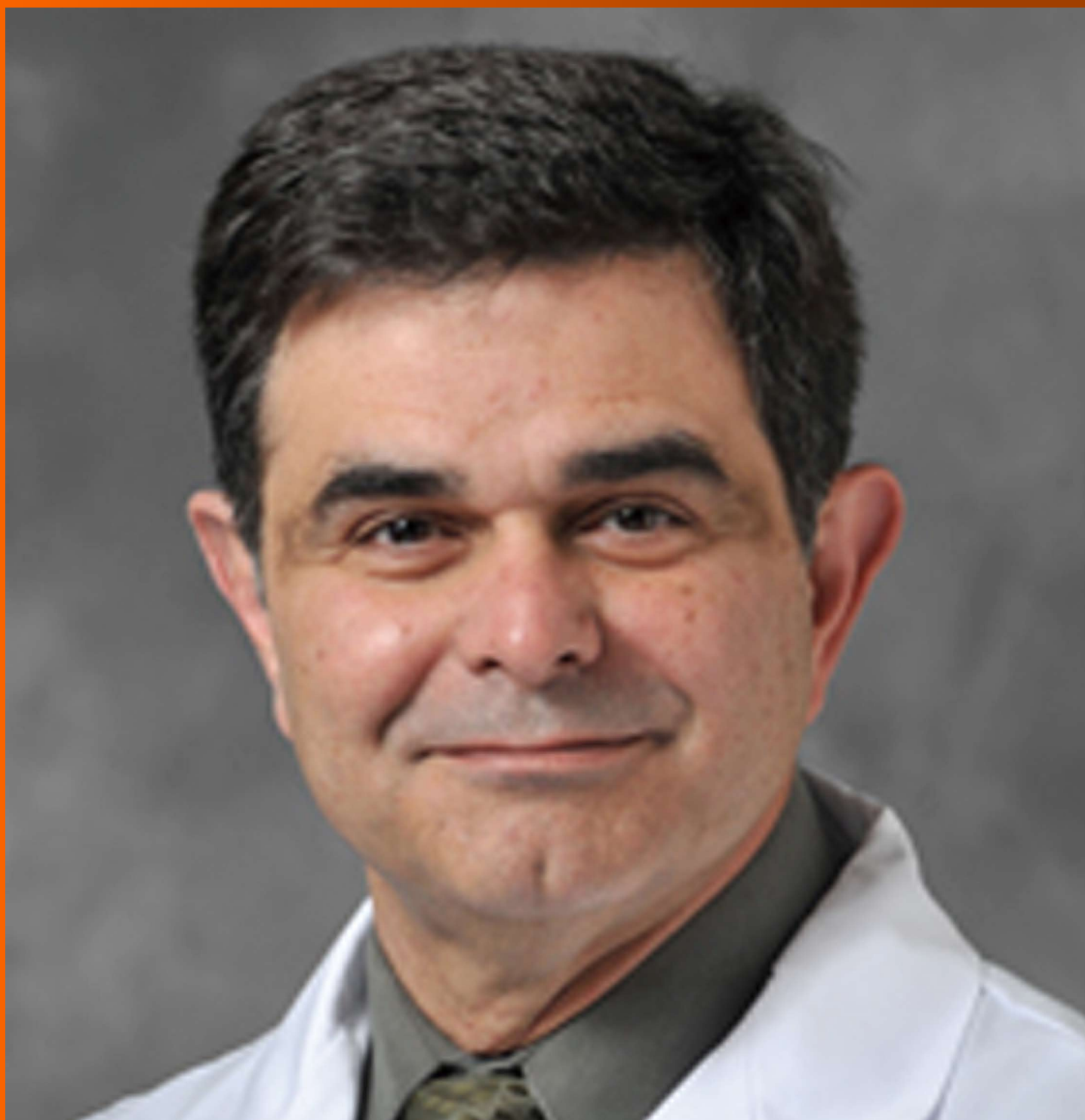
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# World Journal of *Clinical Infectious Diseases*

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## Prosthetic joint infections-a clinico-microbiological perspective: Review article

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### Abstract

Prosthetic joint infections (PJIs), although not very common, currently pose a very significant threat since they are associated with severe complications, high morbidity rates and substantial costs. PJIs are most commonly caused by *Staphylococcus aureus* and coagulase-negative staphylococci. The diagnosis of implant-associated infections is very challenging since no single routinely used laboratory or clinical test has been shown to demonstrate adequate results with respect to sensitivity, specificity and accuracy. In most cases, a sum of clinical signs and symptoms, histopathology, blood tests, radiography, bone scans and microbiological testing is considered to arrive at an accurate diagnosis. Treatment of PJIs is also very difficult since most of the infections are caused by biofilm-producing microorganisms which are significantly more resistant to the hosts natural defense mechanisms and antibiotic treatment. For successful management, a combination of both antibiotic and surgical treatment is most often required, and early diagnosis is of the utmost importance. Thus, a multidisciplinary approach is potentially the best option in dealing with PJI, and should include the involvement of microbiolo-

gists, orthopedic specialists, clinicians, pathologists and radiologists in order to improve decision-making processes and ensure overall success. The following review aims at briefly outlining the microbiology, diagnostic and treatment options, and preventive measures associated with such infections.

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**Key words:** Prosthetic joint infections; Biofilms; Diagnosis; Antimicrobial therapy; Surgical treatment

**Core tip:** Prosthetic joint infections (PJIs), although uncommon, may be associated with significant complications and morbidity. Staphylococci are among the most commonly involved organisms. Diagnosis may be challenging in spite of the availability of various laboratory and radiological tests. Treatment too, is often difficult because most infections are caused by biofilm producing organisms. A combination of prudent surgical intervention and specific antibiotic treatment is the key to a successful management. Thus, a multidisciplinary approach is the best option in dealing with PJI, and should involve a team of orthopedic specialists, clinicians, pathologists, radiologists and microbiologists to ensure best outcomes.

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### INTRODUCTION

The use of prosthetic material in orthopedic surgery has become common in recent times, due to its high success rate with total joint replacement and the management of

fractures. Whilst not very common, prosthetic joint infections (PJIs) pose a very significant threat, considering the number of total joint replacements undertaken each year and the millions of people who currently have indwelling prostheses<sup>[1]</sup>. These infections are associated with severe complications, high morbidity rates and substantial costs<sup>[2]</sup>. PJIs linked with total hip and knee arthroplasty occur at an incidence rate of 1.5%-2.5% in the case of primary interventions. However after revision procedures, rates as high as 2%-20% have also been reported<sup>[3]</sup>.

PJIs are generally classified as per their time of onset after surgery. In the case of early PJI, the first signs and symptoms appear in the initial 3 mo post-surgery. However, there are some authors who are in disagreement with the prior mentioned timeframe, and limit the same to the first 2 to 4 wk. Similarly, in delayed manifestations initial infection signs and symptoms occur anywhere between 3 mo and 2 years post-surgery; and late manifestations, > 2 years post-surgery<sup>[4]</sup>.

## RISK FACTORS

Certain groups of individuals can be considered to be at a higher risk of prostheses infections when compared to others. These include patients those who suffer from rheumatoid arthritis, diabetes mellitus, psoriasis, *etc.*; and those who are immunocompromised, aged, infected with human immunodeficiency virus, have had long-term urinary catheterization, and/or have poor nutritional status<sup>[1,3]</sup>. Other risk factors include smoking, obesity, corticosteroids, burns, liver disease, neoplasia, chemotherapy and radiotherapy<sup>[2]</sup>. In cases where PJI patients possess more than one implant, there is a significant risk of a subsequent infection developing in the other prostheses present at the time of infection<sup>[5]</sup>.

Mortality associated with PJIs in the case of elderly patients is as high as 8%<sup>[6]</sup>. In a study conducted by Grammatico-Guillon *et al*<sup>[7]</sup> in 2012, bone and joint infections were found to be not only age related, but were also found to be higher in males. This parallels the general trend of Rheumatoid Arthritis in the same group of individuals. The study also showed that the most frequent comorbidities associated with such infections are diabetes, ulcer sores and peripheral vascular disorders<sup>[7]</sup>.

## PATHOGENESIS

The development of a PJI is the result of an interaction between the patient, pathogen, environment, intervention and wound factors<sup>[2]</sup>. Implanted devices are known to possess a substantial risk of bacterial and fungal colonization. Studies have shown that having a foreign body present significantly decreases the minimal infecting dose of *Staphylococcus aureus* (*S. aureus*) causing a permanent abscess. In an experiment involving animal models, it has also been shown that as low as 100 colony-forming units of *S. aureus* can be sufficient to infect 95% of subcutaneous implants present<sup>[8]</sup>.

Host defense in the vicinity of implant region also plays a significant role in the emergence of PJIs. The most significant microorganisms responsible for causing these infections are *S. aureus* and coagulase-negative staphylococci (CoNS). It has been found that even few of these skin-derived microorganisms are capable of colonizing implants during surgery, since granulocytes surrounding the implant are rendered incapable of clearing bacteria. This is because granulocytes that accumulate around the implant become partially degranulated due to the introduction of a non-phagocytosable foreign object, and superoxide production is also decreased. After a biofilm has been established, even fully functional granulocytes cannot clear adherent staphylococci<sup>[8]</sup>.

PJIs occur usually by one of two routes, the first being introduced at or about the time of operation. This may be caused by wound sepsis post-operatively, by infected haematomas, or by operative contamination. Early infections may be either due to a single organism (*e.g.*, *S. aureus*) or may be polymicrobial in nature. PJIs caused by CoNS tend to present themselves relatively late (even after a year in some cases). However, even these infections are caused at the time of operation itself. The delayed presentation of such organisms is due to their low pathogenicity and tendency to produce biofilms, which allows them to stay sub clinical for considerable periods of time<sup>[8,9]</sup>. The second route is by that of haematogenous spread. Any existing bacteraemia can lead to prosthetic material infection, although *S. aureus* is considered to be the most common cause. In cases where sinuses are present, PJIs may even involve a range of skin organisms, including Gram-negative, Gram-positive and anaerobic bacteria<sup>[9]</sup>.

## MICROBIOLOGY

Prosthetic material provides an ideal environment in which many microorganisms can flourish<sup>[10]</sup>. The most common bacterial agents responsible for close to 65% of all PJIs are *S. aureus* and *Staphylococcus epidermidis*. This is because these bacteria possess surface proteins that have adhesive properties, facilitating their initial colonization. These microorganisms have been reported in early as well as in late infections associated with total knee and total hip arthroplasty, and are commonly found to be methicillin resistant. Recently, Lee *et al*<sup>[11]</sup> reported that not only is *S. aureus* responsible for causing PJIs, it may also be an independent risk factor that may be responsible for treatment failure. Other microorganisms such as streptococci, diphtheroids and enterococci have been found to be responsible for approximately 10% of PJIs each<sup>[2,3]</sup>.

Gram negative microorganisms are less common than Gram positives in PJIs. However, infections caused by them are far more complicated and require longer treatment<sup>[12]</sup>. Polymicrobial infections have been found to occur usually in early PJIs<sup>[13]</sup>. These, along with infections caused by unusual pathogens such as *Brucella* spp. and various mycobacteria; although not as common, have



also been reported. Anaerobic microbes are found usually only as a part of polymicrobial infections<sup>[2]</sup>. In cases of tubercular PJIs, misdiagnosis proves to be a substantial risk owing to the low index of suspicion attributed to such infections. This could lead to a delay in correct diagnosis, with the risk of permanent damage due to a late treatment<sup>[14]</sup>. PJIs are also caused by fungal agents like *Candida* and *Asperillus*<sup>[15]</sup>.

There are several PJI cases where the growth of microorganisms is absent from aerobic and anaerobic cultures of periprosthetic tissue samples<sup>[3]</sup>. Such culture negativity is an important issue in the treatment of PJIs since negative cultures raise uncertainty in the diagnosis of infection and makes choosing the appropriate antibiotics very challenging<sup>[16]</sup>. A study by Choi *et al*<sup>[16]</sup> showed that the success rate of infection control in the case of culture negative infections is higher, suggesting that the culture negativity may not necessarily be a negative prognostic factor for treating prosthetic joint infections.

Microorganisms found in PJIs that are caused by haematogenous dissemination include Gram negative bacilli, enterococci and anaerobes from genitourinary and gastrointestinal tract procedures or infections; *Streptococci viridans*, *Peptococcus* spp. and *Peptostreptococcus* spp. from dentogingival processes and manipulations; and *Streptococcus* spp. from pyogenic skin infections<sup>[3]</sup>. Few cases of PJIs caused by *Haemophilus parainfluenzae* have also been reported, most caused due to bacteraemia from dental origin<sup>[17]</sup>. Recently, Bjerke-Kroll *et al*<sup>[18]</sup> also reported *Streptococcus viridans* as a significant risk factor for transient bacteremia following dental extractions.

## ROLE OF BIOFILMS

PJIs are characteristically caused by biofilm-forming bacteria. Biofilms can be defined as microorganisms possessing altered phenotypes that live together in a self-organized aggregate. This aggregate is embedded in an exopolymer saccharide matrix which is self-produced and allows the attachment of the biofilm to an external surface. Prosthesis colonization by biofilm-producing bacteria can occur during implantation or by haematogenous seeding. A variety of microorganisms are known to grow in biofilms, including *S. aureus* and CoNS, which are of particular importance in PJIs<sup>[3]</sup>. The ability of microorganisms to form biofilms is a virulence factor<sup>[8]</sup>. Biofilm microbes are considered to be around 10 to 1000 times less susceptible to antibiotics, especially cell wall targeting agents. It is also difficult to predict the pharmacodynamic parameters of antimicrobial agents against these microorganisms. Thus, drugs are often used in concentrations higher than would be otherwise required<sup>[3]</sup>.

There have been several postulations and investigations to explain the increased antimicrobial resistance associated with biofilm-producing bacteria. Although the extracellular matrix in biofilms does physically restrict antimicrobial agents to some extent, it does not seem to be the predominant mechanism conferring resistance. Some

bacteria enter a non-growing stationary state due to decreased nutrient and oxygen levels inside the biofilm. As a result, their susceptibility to antimicrobials is reduced given that their action is growth-dependent. Some bacteria also differentiate into phenotypically resistant states; while others have shown to exhibit gene expression that confers antimicrobial resistance which is biofilm-specific, but has no role in biofilm formation<sup>[3]</sup>.

## DIAGNOSIS

PJIs currently have no universal definition. Most generally, a PJI is considered to be present when at least one of the forementioned criteria is fulfilled-evident purulence in the synovial fluid or around the prosthesis; presence of acute inflammation when periprosthetic tissue sections are histopathologically examined; sinus tract communication with the prosthesis; or when the same organism is found to be growing in repeated cultures of either synovial fluid, periprosthetic tissue or the implant itself<sup>[19]</sup>.

With the evaluation of patients suspected to have PJI, clinicians initially have to verify whether the dysfunction has been septically or aseptically caused. In some cases clinical diagnosis of PJIs is possible; however, laboratory testing is often required. On arriving at a satisfactory diagnosis, clinicians look to microbiological results for the identification of the underlying microbial cause, as well as to attain antimicrobial susceptibility levels so as to execute appropriate treatment. When a PJI is due to a virulent organism such as *S. aureus*, clinical symptoms like fever, effusion, warmth, erythema, and pain localized to the joint may occur. However, if the infection is caused due to low-virulence organisms like *P. acnes* or CoNS, pain and/or loosening of the prosthesis can take place without manifesting any further clinical signs. This leads to a challenging situation where the clinician cannot positively distinguish such an infection from aseptic arthroplasty failure<sup>[19]</sup>. When a PJI is suspected, it is recommended to wait a minimum of 15 d post any antibiotic treatment before performing tests in order to decrease the rate of false negative results, following which pre-operative sampling is to be performed. If the results are positive, surgical and antibiotic management can be planned. In the case of fever and other nonspecific clinical symptoms, it is recommended to perform blood cultures for aerobic and anaerobic bacteria so that rapid probabilistic antibiotherapy can be initiated before considering surgery. Negative results do not rule out the possibility of an existing infection<sup>[15]</sup>.

In the diagnosis of PJIs, no single routine clinical or laboratory test is known to demonstrate ideal sensitivity, specificity and accuracy. Therefore, a sum of clinical signs and symptoms, blood tests, histopathology, radiography, bone scans and microbiological testing is collectively used to attain an accurate diagnosis<sup>[3]</sup>. Blood tests are usually the first laboratory tests to be carried out. In the immediate postoperative period, C-reactive protein levels are found to be elevated and leucocytosis is ob-

served, which returns to normal within a few weeks<sup>[2]</sup>. Thus repetitive measurements can be indicative of PJI, but these results are neither sensitive nor specific<sup>[8]</sup>. In histopathological studies, tissue neutrophil levels are known to suggest infection. However, a generally accepted definition does not exist for acute inflammation. Several imaging techniques are employed to detect PJI. These include General Radiography, computed tomography, Ultrasonography, magnetic resonance imaging, Bone Scintigraphy with Technetium-99m-labelled Methylene Diphosphonate and Fluorodeoxyglucose positron emission tomography<sup>[3,8]</sup>.

## MICROBIOLOGIC ANALYSIS

### Preoperative specimens

This involves the testing of sinus tracts, superficial wounds, synovial fluid and preoperative tissue samples prior to any surgical revision procedure<sup>[19]</sup>. However, cultures of superficial wounds, sinus tracts often represent microbial colonization which can be misleading, and therefore lack predictive value<sup>[3,19]</sup>. Isolation of *S. aureus* from sinus tracts is considered to be predictive of the causative pathogen<sup>[3]</sup>.

Pre-operative culture of Aspirated Synovial fluid can be useful in the diagnosis of PJIs; especially in patients who have underlying inflammatory diseases, where routine Inflammatory markers may not very reliable<sup>[3,19]</sup>. Synovial fluid cultures allow microorganism identification with a sensitivity of as high as 82%-94%, and a specificity of 94%-97%<sup>[3]</sup>. Use of paediatric blood-culture bottles has been shown to improve sensitivity<sup>[8]</sup>. However, reports of discordance between synovial fluid and intraoperative cultures, with rates of false-positives between 3% to 16% and false-negative results from 8% to 50%. It has been shown that the false-negative results can be attributed to the bactericidal effect of anesthetics, low bacterial loads in synovial fluid and presence of fastidious organisms. The instillation of normal saline into joints to aid aspiration may result in the dilution of the specimen bacterial load. Gomez *et al*<sup>[19]</sup> in 2011 stated that preoperative tissue cultures do not suggest any advantage over synovial fluid cultures.

### Intraoperative specimens

Periprosthetic tissue samples are commonly examined to detect infecting microorganisms in PJIs. Prior to sampling, it is imperative to discontinue any antimicrobial therapy for a period of at least 2 wk<sup>[8]</sup>. Gram stains of periprosthetic tissues are rarely given any clinical importance due to their extremely low sensitivity that ranges from 0% to 30%. The sensitivity of bacterial detection from periprosthetic tissue cultures using conventional techniques is between 37% and 61%. This relatively low sensitivity may be attributed to the fact that the growth of the causative microorganisms is more so on the implant surface rather than in the surrounding tissue. To increase sensitivity, it is recommended that the evaluation of a minimum of five

specimens should be carried out<sup>[20]</sup>. Some studies even suggest extending the incubation period for the cultures, and sonication of the tissue samples to disperse adherent bacteria<sup>[3]</sup>. False-positive results are obtained from periprosthetic tissue cultures due to contamination during surgery, specimen transport, or specimen processing, these effect the specificity of results. Moreover, problems with specificity occur due to difficulties in establishing the clinical significance of common skin flora growth in intraoperative cultures<sup>[20]</sup>.

It's a well known fact that Swab cultures are less efficient as compared to tissue and synovial fluid culture samples, and should thus be avoided. They are more prone to contamination and have a high tendency to convey false-positive results. Anaerobes are most commonly isolated using blood culture bottles when compared to isolation from swab and tissue cultures<sup>[20]</sup> thereby indicating loss of bacterial viability during transport that may be responsible for some false-negative culture results.

### Removed implant or fragments

The growth of biofilm-forming bacteria may evade detection using periprosthetic tissue cultures<sup>[20]</sup>. Since biofilms play a very important role in infections, implant cultures has been found to be superior as compared to tissue culture. Explanted foreign material is usually vortexed or sonicated before culturing. This detaches the biofilm-associated bacteria, leaving it liberated in the surrounding broth<sup>[8]</sup>. Sonication has been used to diagnose infections of multiple medical device types, including orthopedic devices, breast implants, vascular grafts, cardiac devices, vascular catheters, and ureteral stents. Sonicate fluid has the advantage over periprosthetic tissue culture in having a shorter time to positivity<sup>[20]</sup>.

### Other non-culture PJI diagnostic techniques

The serological detection of PJI-causing microorganisms involves the detection of antibodies against such organisms. This technique, although fairly simple to perform, lacks substantial specificity which may be attributed to low basal antibody titers that are often recorded against organisms like CoNS, since they are apart of normal human flora. Direct visualization of PJI-associated bacteria from sonication fluid, using immunofluorescence microscopy by means of pathogen-targeted antibodies, can also be used in the diagnosis of PJI. However this method lacks clarity and provides no evident advantage over conventional culture techniques. Molecular techniques, theoretically, have shown to exhibit very good potential in overcoming drawbacks associated with culture methods when it comes to biofilm-associated infections. Unfortunately, studies evaluating these methods are very limited and are often contradictory. The major disadvantages associated with the molecular diagnosis of PJIs are the inability of the technique to provide essential antimicrobial susceptibility results, and the incidence of false-positive results that occur. False-positive results may be caused by DNA from non-viable bacteria that may have

contaminated patient specimens and reagents; or caused by amplicon contamination<sup>[20]</sup>.

## TREATMENT

Surgical intervention and antimicrobial therapy are the two main modes of treatment for the management of PJIs, which may even be initiated while awaiting microbiological results to avoid permanent joint cartilage damage<sup>[10]</sup>. To achieve complete therapy success, a combination of both these methods is usually required, and early diagnosis is of the utmost importance<sup>[8]</sup>.

### Antimicrobial therapy

There are no general standards for an ideal regimen and duration for the administration of antimicrobial agents in the treatment of PJIs<sup>[3]</sup>. In the presence of a prosthetic device, growth recurrence is frequent. Therefore, as in treating tuberculosis cases, killing of all microorganisms is essential<sup>[8]</sup>.

A well-defined example of optimal antimicrobial therapy is the use of Rifampicin in staphylococcal implant infections<sup>[8]</sup>. It has excellent efficacy against staphylococci in its stationary phase, exceeds minimum inhibitory concentrations (MICs) by a factor of about 10-100 at trough levels and can also be well absorbed orally<sup>[3]</sup>. It also has proven *in vitro* activity in several clinical studies. Rifampin must always be used in combination with another antibiotic so as to prevent the emergence of resistance. When used synergistically, it has shown excellent activity against susceptible slow-growing and adherent staphylococci. Quinolones have been found to be excellent combination drugs owing to their good bioavailability, safety and activity<sup>[8]</sup>. Ciprofloxacin is another drug that is commonly used in combination with Rifampicin. Several studies have demonstrated improved efficacy in favour of the rifampicin/ciprofloxacin combination as opposed to ciprofloxacin monotherapy, to be used in the treatment of staphylococcal infections associated with orthopaedic implant devices<sup>[3]</sup>. Rifampicin can also be used with other antimicrobial agents like fusidic acid, cotrimoxazole, trimethoprim/sulfamethoxazole, linezolid and minocycline<sup>[3,8]</sup>. However negative results have been reported when Rifampicin has been used with Levofloxacin orally<sup>[3]</sup>.

Staphylococci, both *S. aureus* and CoNS, have increasingly shown resistance to methicillin over the past few years; and Methicillin-resistant *S. aureus* (MRSA) infections have had a severe impact on PJIs. Patients with MRSA infections in periprosthetic tissue cultures have a higher probability of treatment failure as compared to those with methicillin-susceptible *S. aureus* infections. Also, patients with MRSA PJIs are prone to hospitalization for longer periods of time (median 15 d *vs* 10 d). Intravenous glycopeptides are primarily used to treat PJIs caused by methicillin-resistant Gram-positive bacteria. For MRSA infections postsurgery, continuous outpatient vancomycin perfusion has been used successfully. Prolonged administration of teicoplanin once daily has also appeared to be

relatively efficacious. Newer antibiotics like quinupristin/dalfopristin, linezolid, daptomycin and tigecycline are active against MRSA strains. Drugs like oritavancin, dalbavancin, faropenem and telavancin have shown promising results<sup>[3]</sup>. Quinopristin/dalfopristin shows activity against *Enterococcus faecium* (including vancomycin-resistant strains) as well as *S. aureus* (including MRSA). In a study of 40 patients suffering from MRSA orthopaedic infections and who were put on Quinopristin/dalfopristin, clinical success was found in 78% and microbial eradication in 69% of the cases<sup>[8]</sup>. However, side-effects and drug interactions has halted its use widely<sup>[3]</sup>.

Linezolid shows potential in the treatment of PJIs because of its bioavailability and antimicrobial spectrum. It possesses a very wide anti-Gram-positive bacterial spectrum, which includes all CoNS species. It also shows good diffusion in bone tissue. In an comparative study by Cobo *et al*<sup>[21]</sup>, 86% of patients treated with linezolid demonstrated clinical and microbiological cure. Unfortunately, toxicity associated with the drug is a matter of concern. Adverse effects like reversible myelo suppression, optic neuropathy and peripheral neuropathy are quite common with linezolid treatment. In most cases, optic neuropathies were resolved after linezolid stoppage, but peripheral neuropathies were found to be irreversible<sup>[8]</sup>.

Drugs like Daptomycin and Tigecycline make for attractive options in the treatment of PJIs<sup>[3,8]</sup>. Daptomycin has rapid bactericidal activity against most Gram-positive microbes, including MRSA, Vancomycin-resistant *S. aureus*, and Vancomycin-resistant enterococci<sup>[8]</sup>. Tigecycline is a novel glycylcycline antibiotic possessing a broad spectrum of activity. It has *in vitro* bacteriostatic activity against several Gram-positive, Gram-negative, anaerobic, atypical and antimicrobial-resistant bacteria. However, there is not enough available data for the application of these drugs in PJI management<sup>[3]</sup>.

### Surgical treatment

The management of suspected PJIs mainly rests in the hands of orthopaedic surgeons since surgical procedures are most often required<sup>[22]</sup>. Debridement is usually the first step in surgical treatment and involves the removal of any scar tissue, haematoma, devitalized tissue, and sinus tracts<sup>[2]</sup>. This method has been found to have limited success (varying from 20%-60%); and prosthesis removal and replacement is frequently required<sup>[2,23]</sup>. This however, may be attributed to the fact that debridement and implant retention has often been carried out on patients who have not entirely been suited to the procedure. Recently several algorithms have been published to aid in the selection process, and have been found to significantly increase the success rate of debridement procedures<sup>[23]</sup>. One-stage or Direct Revision is sought to in patients for whom two staged operations would represent a substantial operative risk<sup>[1]</sup>. It involves the replacement of an old prosthesis with a new one during the same surgical procedure<sup>[8]</sup>.

Two-stage (staged) Revision is the commonest surgi-



cal intervention in PJI management<sup>[9]</sup>. Success rates of > 90% have been reported in several studies for the procedure, when 6 wk of antimicrobial treatment have been administered between the stages<sup>[24]</sup>. Recently, Tsai *et al*<sup>[25]</sup> reported that patients who have undergone two-stage revision are less prone to recurrent infections. The surgery is especially preferred in cases where resistant or difficult-to-eradicate microorganisms are involved like MRSA, enterococci, staphylococci, Multi drug-resistant *Pseudomonas aeruginosa* or fungi<sup>[8]</sup>. In the first stage of the procedure, the prosthesis is removed along with carrying out a thorough debridement of all existing dead and infected tissue. The prosthesis is usually replaced by an antibiotic-loaded cement spacer in order to prevent joint space contracture that may occur between stage<sup>[26]</sup>. Other antibiotics may also be administered systemically. In very few cases that involve an acute infection, the implant may be preserved. The second-stage of the procedure involves the insertion of a new prosthesis into the site previously made sterile, and is usually carried out about 8-12 wk after the first stage once all inflammatory markers have attained normal levels and wound healing is complete<sup>[9]</sup>.

Permanent removal of the prosthetic device is usually reserved for patients with a high risk of reinfection, like those with severe immunosuppression or those who are active intravenous drug users; or when no functional improvement is expected after reimplantation<sup>[8]</sup>. Occasionally, in cases where the patient is not suitable for surgical procedures or for those who refuse operations, a long-term suppressive antibiotic regimen may be employed to suppress bacterial growth and to control symptoms<sup>[1]</sup>. However, since this approach only controls clinical symptoms rather than actually curing PJI, the infection tends to relapse in most patients (> 80%) on discontinuing the antibiotics<sup>[8]</sup>.

## PREVENTION

Adequate antibiotic prophylaxis is essential in reducing the risk of prosthesis-associated infections<sup>[10]</sup>. The objective of antimicrobial prophylaxis is to obtain serum and tissue drug levels that exceed the MICs for organisms that are likely to be a threat, for the entire duration of the operation. Systemic antibiotics should be administered just prior to the surgery. While administering prophylactic treatment, it is important to consider antibiotic resistance patterns associated with individual hospitals, as well as toxic and allergic reactions that may occur in patients undergoing surgery. In Orthopedic surgery, first- or second-generation cephalosporins such as cefazolin or cefuroxime are rational choices. However if the patient suffers from a  $\beta$ -lactam allergy, drugs like vancomycin, teicoplanin or clindamycin should be used<sup>[3,8]</sup>. Recently, Qadir *et al*<sup>[27]</sup> suggested Vancomycin to be used as a topical powder in the prevention of PJI. In settings where there is a high prevalence of MRSA, the use of glycopeptides is considered to be appropriate<sup>[8]</sup>.

From the moment of implantation, there is a constant risk of infection due to transient bacteraemia. Haematog-

enous seeding may occur at any point in the lifespan of a patient; however, the risk is significantly higher in the early stages following implantation. It is recommended to maintain good oral hygiene along with the regular removal of dental plaque to reduce the risk of bacteraemia. Also, if any dental, genitourinary tract or gastrointestinal tract procedure is to be undergone post implantation, a single dose of antibiotic prophylaxis should be utilized<sup>[3,8]</sup>.

## CONCLUSION

Prosthetic Joint Infections are still considered to be a significant risk worldwide due to the high mortality risks and exponential costs associated with them. And although several strides have been made to improve their diagnosis and management, numerous obstacles are yet to be tackled. A multidisciplinary approach is potentially the best option in dealing with PJI, and should include the involvement of microbiologists, orthopedic specialists, clinicians, pathologists and radiologists in order to improve decision-making processes and ensure overall success.

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## Reverse genetics: Unlocking the secrets of negative sense RNA viral pathogens

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### Abstract

Negative-sense RNA viruses comprise several zoonotic pathogens that mutate rapidly and frequently emerge in people including Influenza, Ebola, Rabies, Hendra and Nipah viruses. Acute respiratory distress syndrome, encephalitis and vasculitis are common disease outcomes in people as a result of pathogenic viral infection, and are also associated with high case fatality rates. Viral spread from exposure sites to systemic tissues and organs is mediated by virulence factors, including viral attachment glycoproteins and accessory proteins, and their contribution to infection and disease have been delineated by reverse genetics; a molecular approach that enables researchers to experimentally produce recombinant and reassortant viruses from cloned cDNA. Through reverse genetics we have developed a deeper understanding of virulence factors key to disease causation thereby enabling development of targeted antiviral therapies and well-defined live attenuated vaccines. Despite the value of reverse genetics for virulence factor discovery, classical reverse genetic approaches may not provide sufficient resolution for characterization of heterogeneous viral populations, because current techniques recover clonal virus, representing a consensus sequence. In this review the contribution of reverse genetics to virulence factor characterization is outlined, while the limitation of the technique is discussed with

reference to new technologies that may be utilized to improve reverse genetic approaches.

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**Key words:** Reverse genetics; Viral pathogen; Negative sense RNA viruses; Influenza A virus; Ebola virus; Rabies virus; Hendra virus; Nipah virus

**Core tip:** Several negative sense RNA viruses are capricious, pandemic threats and give no quarter to their human hosts. Reverse genetic approaches have been valuable for discovery of key virulence factors mediating disease with the aim of treatment and vaccine development, and knowledge acquisition to genetically map pathogenic potential. Despite the value of the reverse genetics approach current systems are limited by molecular cloning procedures that do not enable reproduction of genetically heterogeneous virus populations that circulate in nature. Advances in molecular biology may facilitate production of genetically diverse viral populations that better represent natural isolates.

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### INTRODUCTION

Rational design of vaccines and antiviral therapies is facilitated by discovery of viral pathogenicity factors, the viral genes and proteins producing disease. Negative-sense RNA viruses are comprised of formidable human and zoonotic pathogens consisting of seven viral families; four are characterized by non-segmented genomes (Filoviridae, Rhabdoviridae, Paramyxoviridae and Bornaviridae).



viridae), while the remaining three are distinguished by segmented genomes (Orthomyxoviridae, Bunyaviridae and Arenaviridae)<sup>[1]</sup>. Before 1994, when pioneering experiments enabled recovery of the first negative-sense RNA virus from cloned cDNA<sup>[2]</sup>, *in vivo* serial virus passage, often at suboptimal temperatures, was the main method utilized to generate pathogenic variants<sup>[3]</sup> and retrospective sequence analysis of viral genes enabled associations between genes and pathogenesis<sup>[4]</sup>. Today reverse genetics is routinely employed to manipulate viral genomes for the purpose of viral pathogenesis research. Briefly cDNAs, representing the full-length RNA genome/genome segments, are cloned into vectors containing T7 RNA polymerase (T7) or RNA polymerase I (pol I) transcriptional units. Transfection of these plasmids, in concert with viral polymerase complex expression, into permissive cells facilitates transcription of viral mRNAs, full length vRNA and recovery of infectious virus<sup>[5]</sup>.

## OPTIMIZING CONDITIONS FOR REVERSE GENETICS

### Minigenome assays

Reverse genetic systems have been optimized for individual viruses by use of minigenomes<sup>[6]</sup>; open reading frames (ORFs) of reporter constructs encoding bioluminescent enzymes or fluorescent proteins are inserted in between viral noncoding sequences that are sufficient for the transcription and replication activity of the viral polymerase<sup>[7]</sup> (Figure 1A). Leader and trailer sequences at the respective 3' and 5' ends of the vRNA are critical for viral polymerase activity, and hence reporter expression, as demonstrated in the case of Marburg virus<sup>[8]</sup> and Ebola virus<sup>[9,10]</sup>. Likewise minigenome constructs for Influenza virus, Orthomyxoviridae, include the 5' and 3' noncoding regions of one of the eight vRNAs such as nucleoprotein (NP)<sup>[11]</sup> or non-structural<sup>[12]</sup> segments. In the case of Paramyxoviruses, however, the addition of gene start and gene end sequences in combination with leader and trailer sequences have been shown to enhance mRNA production<sup>[13]</sup>, while it may also be important for the number of nucleotides of the minigenome to be perfectly divisible by six as each nucleocapsid (N) protein is thought to interact with six nucleotides<sup>[14]</sup>.

For expression of reporter genes from RNA transcripts, produced from minigenome constructs, viral polymerase complexes are supplied *in trans*. Eukaryotic expression vectors such as pCAGGS contain strong promoters such as CAG, chicken  $\beta$  actin fused to a cytomegalovirus enhancer, and transient transfection of polymerase constructs promote sufficient viral protein expression to elucidate the minimum number of viral proteins required for reporter expression. For Influenza virus minigenome activity proteins, which form ribonucleoprotein complexes (RNPs), are required including polymerase basic 2 (PB2), PB1, acidic polymerase (PA) and NP proteins<sup>[15]</sup>. Likewise for Rhabdoviridae<sup>[16]</sup> and Paramyxoviridae<sup>[14]</sup> members plasmids encoding N, phospho-

protein (P) and large polymerase (L) are co-transfected with the minigenome to enable reporter expression. The minimal number of proteins required for minigenome activity may vary considerably even within one virus family, *e.g.*, RNA transcription of Respiratory syncytial virus, Paramyxoviridae, was augmented with the inclusion of matrix (M) 2 protein<sup>[17]</sup>, while addition of M protein to Measles virus (MV) minigenome assays reduced reporter expression by reducing vRNA synthesis<sup>[18]</sup>. Similarly, the expression of accessory proteins may inhibit minigenome activity and repression of some of these proteins may be required for measurement of any polymerase activity<sup>[19]</sup>. The need for different protein combinations for reporter activity underscores the importance of minigenome assays in determining functional associations between viral proteins for viral mRNA transcription.

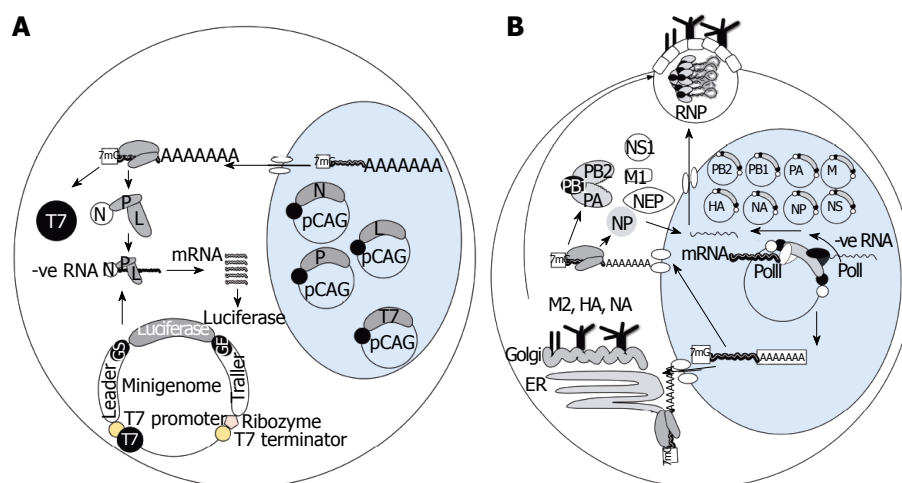
### Poll and II systems: Transcription in the nucleus

Selection of promoters that drive RNA transcription from minigenome constructs is dependent upon whether viral transcription occurs in the nucleus or the cytoplasm during natural replication of the virus. T7-dependent systems may be more suitable for viruses that replicate RNA within the cytoplasm, while polII systems may better mimic viral replication cycles that involve transcription in the nucleus, however recent studies have indicated some exceptions to this view. Infectious Uukuniemi<sup>[20]</sup>, Influenza<sup>[21]</sup>, Thogoto<sup>[22]</sup>, Borna disease virus, MV<sup>[23]</sup> and Ebola virus<sup>[10]</sup> have all been successfully recovered from cloned cDNAs by the use of cellular RNA polymerases such as pol I (Figure 1B). The conventional role of pol I is to transcribe ribosomal RNAs without addition of 5' caps and 3' poly-A tails<sup>[24]</sup>, therefore it is a suitable host enzyme for the processing of viral RNA molecules generating well defined vRNA 3' and 5' termini<sup>[25]</sup>. To employ RNA pol I a cDNA copy corresponding to each viral segment, or a full length cDNA molecule, is placed between a pol I truncated promoter and a pol I terminator enabling synthesis of vRNA<sup>[26]</sup>. Interaction between RNA pol I and its promoter is species-specific, therefore promoter sequence is carefully selected to suit the cell line destined for virus rescue<sup>[27,28]</sup>.

RNA pol II cytomegalovirus promoters have also been utilized to initiate transcription of viral messenger RNA for Influenza virus rescue systems<sup>[26]</sup> and also has been shown to enhance cRNA expression for MV recovery in relation to other reverse genetic systems, dependent upon T7, however as pol II transcripts may be spliced and polyadenylated the utility of pol II for virus rescue of other negative-sense RNA viruses is still to be determined<sup>[23]</sup>.

### T7 systems: Transcription in the cytoplasm

T7 polymerase has been particularly useful for recovery of negative-sense RNA viruses, which mostly undergo transcription in the cytoplasm, including Hendra<sup>[29]</sup>, Nipah<sup>[30]</sup>, MV<sup>[31]</sup>, Sendai<sup>[32]</sup>, Rabies<sup>[32]</sup>, Ebola<sup>[33]</sup>, Marburg<sup>[34]</sup>, Newcastle disease virus<sup>[35]</sup>, RSV<sup>[17]</sup>, Vesicular stomatitis vi-



**Figure 1 Reverse genetic approaches for the study of viral pathogenesis.** This figure demonstrates a T7 driven minigenome system (A) and a poll dependent virus rescue system for influenza A virus (B). In (A) the pCAGGS plasmids enable synthesis of T7 polymerase and the viral polymerase complex; nucleoprotein (N), phosphoprotein (P) and large polymerase (L). T7 drives transcription of the minigenome for intracellular synthesis of negative-sense RNA (-veRNA) genome analogues. The minigenome contains Luciferase flanked by leader and trailer sequences that contain gene start (GS) and gene end (GE) sequences essential for polymerase function. To ensure correct processing of the trailer sequence the hepatitis  $\delta$  ribozyme is included prior to the T7 terminator sequence. The viral proteins encapsidate the RNA analogue and facilitate transcription, which results in Luciferase production and increase in luciferase indicates minigenome transcription. In (B) eight plasmids PB1, PB2, PA, HA, NP, NA, M, NS, representing the influenza A virus genome, are transfected into 293T cells and each of the negative sense viral RNA segments (-veRNA) are produced by poll driven transcription. From the same plasmid, viral messenger RNAs are transcribed in an ambisense direction by pollI. Capped and polyadenylated mRNAs are exported from the nucleus into the cytoplasm. Some viral mRNAs are spliced before export, such as M2 and NEP, and translation of viral mRNA occurs on ribosomes attached to endoplasmic reticulum or on ribosomes free in the cytoplasm. Viral proteins in the cytoplasm return into the nucleus and facilitate replication of the viral genome, which is transcribed to produce more viral mRNA and exported from the nucleus. Viral membrane proteins are modified and transported to the apical membrane of the cell. The RNPs are transported to the plasma membrane containing HA and NA in association with M1 and NEP, and 8 RNPs are packaged into progeny virions for neuraminidase-mediated release. HA: Hemagglutinin; NA: Neuraminidase; PB: Polymerase basic; PA: Acidic polymerase; RNPs: Ribonucleoprotein complexes; NS: Non-structural.

rus<sup>[36]</sup> and Lymphocytic choriomeningitis virus<sup>[37]</sup>. Investigators have had more success in virus recovery by insertion of full-length antigenome, rather than genome sense, between T7 promoter and terminator sequences<sup>[2]</sup>. The T7 promoter can be modified to enhance transcription initiation and reporter expression in minigenome assays by the addition of more than two G nucleotides<sup>[38]</sup>, while if added in combination with a nuclear localization signal the T7 system has enabled recovery of Influenza virus<sup>[39]</sup>. The disadvantage of T7 systems, in contrast to pollI, includes necessary sequences bordering the 5' and 3' antigenome ends to form autocatalytic ribozymes that cleave nonviral terminal nucleotides added during transcription. Early rescue systems focussed on correct processing of the RNA 5' ends, or trailer sequence<sup>[38,40]</sup>, by insertion of an adjacent Hepatitis delta virus (HDV) sequence before the T7 terminator sequence, and the HDV ribozyme sequence has recently been optimized for more efficient vRNA cleavage<sup>[41]</sup>. Enhanced recovery of infectious virus has also been documented by addition of a hammerhead ribozyme sequence prior to the 3' leader<sup>[41]</sup>.

An advantage of T7 dependent systems includes transfectable cell lines of several species can be employed for the purpose of virus recovery, providing supplementation with an exogenous source of T7<sup>[39]</sup>. Choice of cell may be of value when vaccine approved cell lines must be used, or in the case of zoonotic viruses that have limited cell tropism. In early reverse genetics systems cytoplasmic T7 was supplied by addition of recombinant

vaccinia virus<sup>[42]</sup>, however its cytopathic effects have been found to impede virus recovery and necessitates plaque purification for removal of vaccinia from the virus culture<sup>[43]</sup>. These issues have been overcome by use of modified vaccinia Ankara strains<sup>[44]</sup>, however more practical systems are now accessible such as T7 expression plasmids that can be transiently transfected<sup>[45]</sup> or stably transfected<sup>[46,47]</sup> into permissive cell lines.

## REVERSE GENETICS AND PATHOGENICITY FACTOR DISCOVERY

### Influenza A virus

Influenza A virus, a member of *Orthomyxoviridae* family, contains eight negative-sense RNA segments each corresponding to one of eight viral genes; two of these, the hemagglutinin (HA) and neuraminidase (NA) encode the surface glycoproteins that protrude from the viral envelope. Currently 17 HA and 10 NA types have been identified and all but the most recently described subtype (H17N10 from bats) have been isolated from aquatic birds such as waterfowl and shorebirds, which act as a natural reservoir for the virus<sup>[48,49]</sup>. The only subtypes circulating in humans, H3N2 and H1N1, cause mild disease associated with viral replication in the upper respiratory tract and large airways<sup>[50,51]</sup>, while replication in the lower respiratory tract<sup>[52]</sup> or sites outside the respiratory tract results from infection with virulent isolates, such as

**Table 1** Outline of viral proteins that contribute to virulence as determined by reverse genetics

Virus	Virulence factors	Role in pathogenesis
Influenza A	Multibasic cleavage site	Facilitates viral spread to cells outside the respiratory tract
Ebola	Glycoprotein	Transmembrane form mediates host cell attachment and its soluble forms activate mononuclear phagocytes and endothelial cells
	Viral protein 24 and nucleoprotein	Antagonists of IFN responses
	Viral protein 35	Viral polymerase cofactor that suppresses RIG-I like receptor signalling
Rabies	Glycoprotein	Neurotropic surface glycoprotein that facilitates spread to the brain
	Phosphoprotein	Viral polymerase cofactor and antagonist of IFN responses
Hendra and Nipah	Phosphoprotein	Viral polymerase cofactor and antagonist of IFN responses
	V and W proteins	Antagonists of IFN responses
	C protein	Regulates viral transcription and affects activation of innate immune cells

IFN: Interferon.

highly pathogenic avian isolates that infect humans *via* inter-species transmission events<sup>[53]</sup>.

The virulence factors enabling viral spread beyond the respiratory tract have been characterized in reverse genetic studies. Influenza virus reverse genetic systems have been thoroughly optimized since their initial iterations when purified RNP and RNA were transfected *in vitro* and recovered with the addition of helper viruses<sup>[54]</sup>. In early plasmid-based reverse genetic systems influenza viral RNA synthesis was dependent upon supplementation of additional expression plasmids for NP and the polymerase complex, PB1, PB2 and PA, *in trans*<sup>[21,55]</sup>, however shrewd optimization by inclusion of an RNA pol II transcriptional unit on the same plasmid as the RNA pol I promoter, in an ambisense direction, generated viral mRNA molecules *in cis* enabling production of viral protein and vRNA from a single plasmid<sup>[56]</sup>. More recently gene segments were concatenated onto a single cassette that encoded multiple segments each separated by a transcriptional unit with the aim to hasten the recovery of vaccine seed viruses<sup>[57]</sup>, which is essential for production of pandemic vaccines.

One particular virulence factor that has been well characterized in reverse genetic studies includes the HA glycoprotein, which interacts with terminal sialic acids for host cell attachment<sup>[58]</sup> and orchestrates fusion of the viral envelope and endosomal membrane of the cell for release of RNPs into the cytoplasm<sup>[59]</sup>. For efficient fusion the HA precursor must be cleaved at a prominent loop to form two subunits, HA1 and HA2, cleavage of the HA glycoprotein is a process essential for multiple rounds of viral replication and is carried out by enzymes that are produced by the host<sup>[60,61]</sup>. For most human seasonal influenza and Low Pathogenicity Avian Influenza viruses the cleavage occurs at the site of a single arginine (R) residue<sup>[60,62]</sup>. For this reason, these viruses are limited to tissues that contain host enzymes with the corresponding recognition preference for single basic amino acids<sup>[60,63]</sup>.

In the HA of highly virulent subtypes insertions of multiple basic amino acid residues have been found and this region has been coined the multi-basic cleavage site (MBCS, Table 1)<sup>[64,65]</sup>. The role of the MBCS in pathogenesis for chickens was assessed by generating H5N2

mutant viruses containing variable sequence lengths and combinations of basic amino acid residues in the HA *via* reverse genetics<sup>[66]</sup>. In this study they found association between presence of a > 4 basic amino acid residues in the cleavage site, efficient HA cleavage in chicken embryonic fibroblasts and lethality to chickens, which was caused by spread of virus to brain *via* neurons and systemic organs *via* the blood stream. More recent plasmid based reverse genetic studies have demonstrated a similar role for the MBCS in viral pathogenesis for mammals, such as mice<sup>[67]</sup> and ferrets<sup>[68,69]</sup>, which corresponds to the pathological and clinical observations of humans infected with highly pathogenic influenza such as detection of virus in systemic organs of fatal H5N1 cases<sup>[70]</sup> and detection of vRNA in the blood stream of infected patients<sup>[71]</sup>. Since the importance of the MBCS for influenza pathogenesis has been established recent research has focused on reducing replication of Influenza virus with substrate-analogue peptide mimetic inhibitors that target host cell proteases, specifically those carrying out HA0 cleavage<sup>[72]</sup>. This is a successful example of the use of reverse genetics for identification of a virulence factor, the MBCS, and production of an inhibitor based on the understanding of virulence mechanisms.

### Ebola virus

In 1976 Ebola virus, a single-stranded negative-sense RNA virus of 18.9kb within the Filoviridae, first emerged in humans and thereafter several outbreaks in Sudan, western and central Africa have been documented<sup>[73]</sup>. Index cases are often associated with butchering, handling or consuming bush meat such as fruit bats<sup>[74]</sup>, the potential natural reservoir of the virus<sup>[75]</sup>, and also close contact with non-human primates<sup>[76]</sup>. Human-to-human transmission occurs *via* close contact<sup>[77]</sup> and long incubation periods, prior to symptom development, facilitate viral spread in the community causing stigmatization of health care workers and relatives of the sick<sup>[78]</sup>. Ebola viruses isolated from different geographical locations in Africa have caused similar disease symptoms and signs such as headache, myalgia, muscle spasms, fever, malaise, abdominal pain, haemorrhage and maculopapular rash<sup>[79]</sup>, although the latter was more commonly noted in infected patients



of the Zaire outbreak, which of all the outbreaks, has the highest documented case fatality rate<sup>[80,81]</sup>. In contrast humans infected with the Reston Ebola virus isolate, which emerged *via* importation of infected monkeys from the Philippines into Reston, Virginia, United States, developed antibodies to the virus in the absence of clinical disease<sup>[82]</sup> indicating this virus isolate was not pathogenic, although only a small number of humans were exposed. Reston Ebola virus has since been detected in piggeries in the Philippines while serological studies suggest a small number of pig farm workers have been infected with the virus<sup>[83]</sup>.

Both T7 and pol I dependent systems have been utilized for minigenome assays to characterize the viral proteins mediating transcription for Zaire and Reston Ebola viruses, and to recover infectious viruses for several Ebola virus isolates<sup>[84]</sup>. Reverse engineered viruses have been used to assess the role of the Ebola virus glycoprotein (GP, Table 1), which forms trimeric spikes on the viral envelope and mediates host cell attachment and entry<sup>[85]</sup>. Produced through a process of transcriptional RNA editing<sup>[86]</sup>, GP protein expression is regulated in infected cells<sup>[87]</sup>; secretory GP is produced from unedited transcripts, while transmembrane GP is produced from edited transcripts that preside at lower frequency<sup>[88]</sup>. GP expression is cytotoxic acting to increase the permeability of venous and arterial blood vessels, compromising vascular function<sup>[89]</sup>. Recently chimeric Ebola viruses, in which the GP of Zaire and Reston virus isolates were exchanged, have been utilized to clarify isolate-specific differences in virulence<sup>[90]</sup>. Interferon- $\alpha/\beta$  receptor knock-out (IFNAR<sup>-/-</sup>) transgenic mice were selected to characterize *in vivo* pathogenicity of the chimeric viruses, as the need for virus adaptation *via* serial passage is unnecessary, unlike immune competent mice. Reverse engineered Reston virus was not pathogenic to IFNAR<sup>-/-</sup> mice and only replicated to low levels in liver and spleen, which mirrors the absence of human disease<sup>[90]</sup>. In contrast, Zaire virus caused rapid weight loss and replicated to high titres in spleen and liver, which indicated that the IFNAR<sup>-/-</sup> mouse model recapitulated human disease observations in relation to both virus isolates<sup>[90]</sup>. Interestingly, introduction of the Reston GP into the Zaire virus did not attenuate virulence in IFNAR<sup>-/-</sup> mice, suggesting that Zaire GP is not the only determinant of virulence, and instead the robust replicative capacity of Zaire virus *in vitro* and *in vivo* was consistent with its virulence for IFNAR<sup>-/-</sup> mice<sup>[90]</sup>.

Other factors for Zaire Ebola virus virulence have been identified *via* serial passage of reverse engineered viruses in mice and include viral protein 24 and nucleoprotein (Table 1), factors that are involved in evasion of host type 1 interferon responses<sup>[91]</sup>. Other components of the innate immune response, particularly transcription factors such as interferon regulatory factor 3, may be suppressed by viral protein 35 (VP35, Table 1), a cofactor of Ebola viral polymerase<sup>[92]</sup> and production of reverse genetic viruses containing mutations in VP35 have been used in

transcriptome studies<sup>[93]</sup>, which may provide key insights into virus host interactions.

### Rabies virus

Rabies virus, a member of the Rhabdoviridae, was the first single-stranded negative-sense virus recovered by reverse genetics<sup>[2]</sup>. Wildlife host reservoirs of rabies virus include bats<sup>[94]</sup>, raccoons and foxes<sup>[95]</sup> although cross-species transmission to non-human primates and domestic animals such as dogs and cats perpetuate human disease<sup>[96-98]</sup>. Risks for human infection include direct exposure to saliva shed from rabid animals occurring *via* animal bite or contamination of broken skin<sup>[99]</sup>. Human disease results from fatal encephalitis that progresses as virus spreads to the central nervous system *via* retrograde transport along axons of peripheral nerves, which is mediated by rabies glycoprotein (G, Table 1)<sup>[100]</sup>. The 12kb long genome encodes five proteins; nucleoprotein (N), phosphoprotein (P, Table 1), matrix protein (M), G and the RNA-dependent RNA polymerase (L). Viruses produced by reverse genetics have elucidated that G, M and P proteins play important roles in the severity of rabies disease by either facilitating cell to cell spread<sup>[101]</sup> or antagonizing host innate immune responses<sup>[102]</sup>.

Currently rabies virus vaccines delivered to humans are beta-Propiolactone inactivated and administered intramuscularly in three doses to generate neutralizing G-specific antibody titres, which wane overtime, as such additional booster shots may be needed<sup>[103]</sup>. Due to the requirement for multiple doses and the high cost of vaccination and post-exposure prophylaxis (PEP) therapies more than 55000<sup>[104]</sup> rabies-related deaths are still reported annually predominantly in developing countries. Replication deficient rabies viruses (RDRV) produced *via* reverse genetics may be a low cost alternative to current vaccines and PEP. RDRV have been produced by T7 driven reverse genetic systems such that the viruses contained neither coding capacity for M nor P, instead the latter encoded two copies of G for protein over-expression and induction of greater G specific immune responses<sup>[105]</sup>. RDRV vectors have also been shown to be immunogenic demonstrated by the induction of neutralizing G-specific antibodies in non-human primates following a prime boost immunization strategy<sup>[106]</sup>. Safety is a main concern for the use of live attenuated viral vaccines, but because RDRV vectors replicate in cell culture only in the presence of P or M protein supplementation and are innocuous to immune deficient transgenic mice<sup>[106]</sup> the risk of reversion may not be as great as other live attenuated vaccine viruses that are attenuated through single amino acid changes. Rabies virus has also been shown to be a safe and excellent vaccine vector with the ability to generate antibody responses targeting human immunodeficiency virus envelope proteins, severe acute respiratory syndrome coronavirus and hepatitis C virus proteins<sup>[107]</sup>. Collectively, these RDRV studies have highlighted the diverse utility of reverse genetics, not only enabling discovery of virulence determinants, but

also applying our understanding of virulence to rationally engineer attenuated viruses for the purposes of vaccination.

### Hendra and Nipah viruses

Hendra virus (HeV) and Nipah virus (NiV), classified within the Henipavirus genus of the Paramyxoviridae, which are harboured asymptotically by Pteropid fruit bats<sup>[108]</sup>, were identified as aetiological agents of severe human infections that occurred in the 1990's. Inter-species transmission occurs *via* intermediate hosts; infected horses were associated with HeV human cases in Australia<sup>[109]</sup> and infected pigs were associated with NiV cases in Malaysia<sup>[110]</sup>. Additional risk factors for contraction of Nipah virus in Bangladesh have included consumption of raw date palm sap, a delicacy in Bangali culture, contaminated by bat urine or saliva<sup>[111]</sup>, while limited human-to-human transmission has been documented in cases of very intimate contact such as preparing an infected corpse for burial<sup>[112]</sup>. In humans HeV and NiV infections are associated with high case fatality rates and severe disease. NiV infected humans develop respiratory and neurological signs such as dyspnoea, disorientation, confusion and muscle spasms that are associated with the expansive tissue tropism of the virus, which includes infection of neurones of the central nervous system, endothelium, lymphoid and respiratory tissues<sup>[113]</sup>. In 10% of infected people NiV has been shown to reside in a quiescent form for months or years until the virus reactivates causing fatal neurological disease<sup>[114]</sup>. HeV disease in humans has not been thoroughly characterized as few human infections have occurred, however from limited reports of post-mortems and disease signs it appears the respiratory and neurological disease caused by HeV is akin to that caused by NiV infection<sup>[115,116]</sup>.

NiV and HeV have non-segmented genomes of ~18kb in length that encodes for more than six proteins and NiV and HeV are closely related to each other with amino acid sequence similarities of > 80% for many of the viral proteins<sup>[117]</sup>. Nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), glycoprotein (G) and large polymerase (L) are encoded on discrete transcriptional units<sup>[118]</sup>, while three accessory viral proteins are produced from the P gene (Table 1) including the C protein that is transcribed from an alternate open reading frame (ORF), and also V and W proteins that are produced by the addition of G nucleotides into transcribed mRNAs *via* RNA editing<sup>[119]</sup>. P, V, W and C proteins play important roles in infection by impeding activation of host antiviral responses. A conserved feature of the paramyxovirus V protein is its ability to bind melanoma differentiation-association gene 5 (MDA5), a pattern recognition receptor, impeding the recognition of dsRNA resulting in inhibition of IFN- $\beta$  induction<sup>[120,121]</sup>. Furthermore, V and W proteins can prevent activation of the type I IFN signalling pathway by sequestration of signal transducer and activator of transcription (STAT) in the cytoplasm or nucleus, reducing STAT mediated induction of interferon stimulating genes key to innate antiviral

responses<sup>[122]</sup>. Inhibition of various components of the antiviral response by several NiV and HeV proteins underscores the role of P, V, W and C for viral pathogenesis.

HeV and NiV reverse genetics systems have been successful in virus recovery with use of T7<sup>[29,30]</sup> dependent systems and co-transfection of the protein expression plasmids N, P and L, which encapsidate the RNA forming the RNP complex, as these proteins are essential for minigenome function<sup>[14]</sup>. The transfection ratios of N:P:L require optimization for efficient virus recovery as poor reporter expression in minigenome assays have been noted in the context of high concentrations of P protein<sup>[45]</sup>, which likely results from the C protein inhibiting minigenome expression<sup>[19]</sup>. To further examine importance of C, V and W proteins for NiV pathogenesis several recombinant viruses were produced; Stop codons were introduced downstream of the C ORF site or the RNA editing site to prevent expression of C or V and W respectively. Despite these changes all viruses expressed functional forms of the P protein<sup>[123]</sup>. Following confirmation of P but not V, W or C expression in infected cells, it was determined that the recombinant viruses replicated efficiently *in vitro*, which indicated these proteins were not essential for viral replication. Virus pathogenicity was assessed by use of a hamster infection model, wherein it was demonstrated that suppression of C and V, but not W protein, completely attenuated NiV as weight loss, disease signs and high levels of viral replication in systemic organs were not observed<sup>[123]</sup>. Another study by a different group compared host responses in human endothelial cell lines infected with wildtype NiV versus one of the attenuated viruses, NiV lacking C protein expression (NiV $\Delta$ C). With microarray analysis they established that compared to wildtype NiV, NiV $\Delta$ C induced higher levels of cytokines and chemokines such as interleukin 1 beta (IL-1 $\beta$ ), IL-8, CXCL2, CXCL3, CXCL6 and CCL20<sup>[124]</sup>. These findings indicated that NiV C plays a role in inhibiting induction of proinflammatory cytokines and recruitment of leucocytes and lymphocytes into sites of infection such as the lung. This study also examined the pathogenesis of NiV $\Delta$ C in hamsters and the researchers were only able to partially replicate the attenuated phenotype of NiV $\Delta$ C, as 30%-90% of animals required euthanasia following infection. The reason for the variation in results between the two studies is yet to be ascertained, however it highlights the limitations associated with insertion of silent restriction sites for the purposes of engineering mutations into plasmids, as laboratories will insert a different variety of silent mutations that may have unknown effects on the virulence of the virus.

## REVERSE GENETICS TECHNOLOGIES HAVE LIMITED UTILITY FOR THE STUDY OF DIVERSE RNA VIRUS POPULATIONS

RNA viruses, HIV and Influenza virus in particular, spread between and within hosts as genetically hetero-



geneous virus populations, or quasispecies, clustering around a dominant virus sequence<sup>[125]</sup>. Quasispecies are perpetuated by spontaneous mutations afforded by low fidelity viral RNA polymerases, and although exact mutation rates may differ for each virus<sup>[126]</sup>, they are in the range of  $10^{-4}$  mutations per nucleotide copied<sup>[127]</sup>, therefore 1.3 mutations would be expected to occur with every replication of the influenza genome of ~13kb. Quasispecies are thought to act cooperatively with the aim of facilitating viral persistence within hosts<sup>[125,128]</sup>. Genetic heterogeneity has been found important for poliovirus pathogenesis as demonstrated by the ability of a genetically diverse, but not a homogenous, virus population for invasion into the CNS<sup>[129]</sup>. HIV genetic diversity may also influence viral tropism and larger sequence diversity has been associated with faster disease progression<sup>[128]</sup>. With this in mind we are faced with a technological drawback; the requirement of producing infectious clones for the purpose of virus rescue also removes population heterogeneity that may play pivotal roles in pathogenesis. Overall, care should be taken in the selection of a consensus sequence to produce infectious clones representative of dominant and also subdominant variants.

## CONCLUSION

Reverse genetic technologies have proven critical to study the contribution of viral genetic factors to disease severity by enabling production of well-defined, recombinant negative-sense RNA viruses, such as a mutant and wild-type viruses, which can be compared for the purpose of identifying chief virulence determinants in the context of host-pathogen systems. For several negative-sense RNA viruses effective rescue methods have been developed, which may be dependent upon either T7 or pol I and II transcriptional units. Furthermore, inclusion of polymerase proteins or 5' and 3' cleavage sequences for correct vRNA processing may also be necessary for rescue, although these conditions are optimized for each virus and minigenome assays have proved useful for this purpose.

Recombinant viruses, however, are produced by selection of a consensus sequence that forms the basis of the infectious clone and therefore recombinant viruses are likely to constitute only the dominant viral species of a potentially diverse natural virus population. Reduction and alteration in viral heterogeneity, as a consequence of reverse genetics, is a limitation not often taken into account in the context of pathogenesis studies. However, with the advent of next generation sequence technologies for thorough characterization of virus populations we stand in good stead to gather a better grasp of viral heterogeneity in a field isolate and molecular biologists may be capable of recapitulating diverse viral populations *via* reverse genetics. Recent technologies such as Gibson cloning<sup>[130]</sup> and barcoding virus populations<sup>[131]</sup> are likely to enable researchers to produce heterogeneous virus populations that can be studied for characterization of

the pathogenic potential of diverse viral populations, with a particular focus on the importance of subdominant viruses for severe disease outcomes. Despite this limitation, reverse genetics enables production of viruses that may be utilized for various future applications such as live-attenuated vaccines, mapping neural pathways in the brain, oncolytic virus production and delivery of microRNAs as a therapy for viral infections.

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## Subversion of cellular stress responses by poxviruses

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### Abstract

Cellular stress responses are powerful mechanisms that prevent and cope with the accumulation of macromolecular damage in the cells and also boost host defenses against pathogens. Cells can initiate either protective or destructive stress responses depending, to a large extent, on the nature and duration of the stressing stimulus as well as the cell type. The productive replication of a virus within a given cell places inordinate stress on the metabolism machinery of the host and, to assure the continuity of its replication, many viruses have developed ways to modulate the cell stress responses. Poxviruses are among the viruses that have evolved a large number of strategies to manipulate host stress responses in order to control cell fate and enhance their replicative success. Remarkably, nearly every step of the stress responses that is mounted during infection can be targeted by virally encoded functions. The fine-tuned interactions between poxviruses and the host stress responses has aided virologists to understand specific aspects of viral replication; has helped cell biologists to evaluate the role of stress signaling in the uninfected cell; and has tipped immunologists on how these signals contribute to alert the cells against pathogen invasion

and boost subsequent immune responses. This review discusses the diverse strategies that poxviruses use to subvert host cell stress responses.

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**Key words:** Poxvirus; Cell stress response; Heat shock response; Chaperones; Unfolded protein response; Host translational control; Hypoxia; Oxidative stress; DNA damage

**Core tip:** Poxviruses are known to encode a plethora of proteins that interact with cell biology processes in order to achieve replicative success. In this article, we review how poxviruses cope with cellular stress signals that are usually triggered upon infection to tentatively block virus replication. The understanding of mechanisms by which poxviruses and other complex viruses interfere with stress responses can further illuminate the web of pathways regulating cell homeostasis, as well as how viruses intertwine their own biochemical needs into this intricate scenario.

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### INTRODUCTION

The Poxviridae family is taxonomically divided into two subfamilies of double-stranded DNA (dsDNA) viruses that are able to infect insects (Entomopoxvirinae) and a wide spectrum of vertebrate hosts (Chordopoxvirinae). The Chordopoxvirinae subfamily currently contains ten genera (Avipoxvirus, Capripoxvirus, Cervidpoxvirus, Crocodylidpoxvirus, Leporipoxvirus, Molluscipoxvirus, Orthopoxvirus, Parapoxvirus, Suinopoxvirus, Yatapoxvirus) and one unassigned species (Squirrelpox virus), whereas the Entomopoxvirinae subfamily comprises three genera

(Alphaentomopoxvirus, Betaentomopoxvirus, Gammaentomopoxvirus) and two unclassified species (*Diachasmimorpha entomopoxvirus* and *Melanoplus sanguinipes entomopoxvirus* “O”)<sup>[1]</sup>. Members of the Poxviridae family are large viruses (approximately 350 nm × 250 nm × 200 nm) with a linear genome ranging from 130 to 300 kb, each often encoding approximately 200 proteins. Virions are brick-shaped, multi-enveloped particles and, unlike other DNA viruses, replicate exclusively in the cytoplasm of the infected host cell. Most poxviral biosynthetic pathways occur in distinct sites of the cytoplasm called viral factories: large masses of electron dense material, the viroplasm, that are frequently surrounded by membranes from the endoplasmic reticulum (ER) and/or membranes from the ER-Golgi intermediate compartments<sup>[2-6]</sup>.

Different viruses have evolved two very distinct general strategies to compete with the host cell for biochemical resources and successfully replicate within them. One such strategy is a “hit and run” type of approach, in which viruses rapidly replicate and generate a progeny that spreads quickly to other cells. In order to be effective, these viruses have invested in replication speed by keeping small genomes which code for few essential proteins - the faster they replicate, the more efficiently they can escape antiviral responses by the host. A second strategy, however, is based on a “stay and fight” approach. Viruses that adopted this strategy tend to endure within the host cell and, therefore, may be susceptible to antiviral responses that are gradually elicited against them. Thus, in order to achieve replicative success, these viruses have to cope with the host attempts to get rid of them and, as a way to counteract antiviral responses, many evolved processes to either block or delay such responses. Because most viral strategies to evade host responses are based in virus-coded proteins, this led inevitably to an increase in genome sizes. There are obvious exceptions to this rather simplistic classification of virus replication strategies, as in the case of hepadnaviruses (like hepatitis B virus) for instance. Nonetheless, most viruses can still fit one of the two aforementioned models. Poxviruses are one of the best examples of viruses that have developed ways to either counteract host strategies to hamper viral replication or boost their biosynthetic pathways to the detriment of the host's. Indeed, most poxviruses (especially chordopoxviruses) spare up to 50% of their genomes to code for immune evasion-related and host-interaction genes<sup>[7]</sup>.

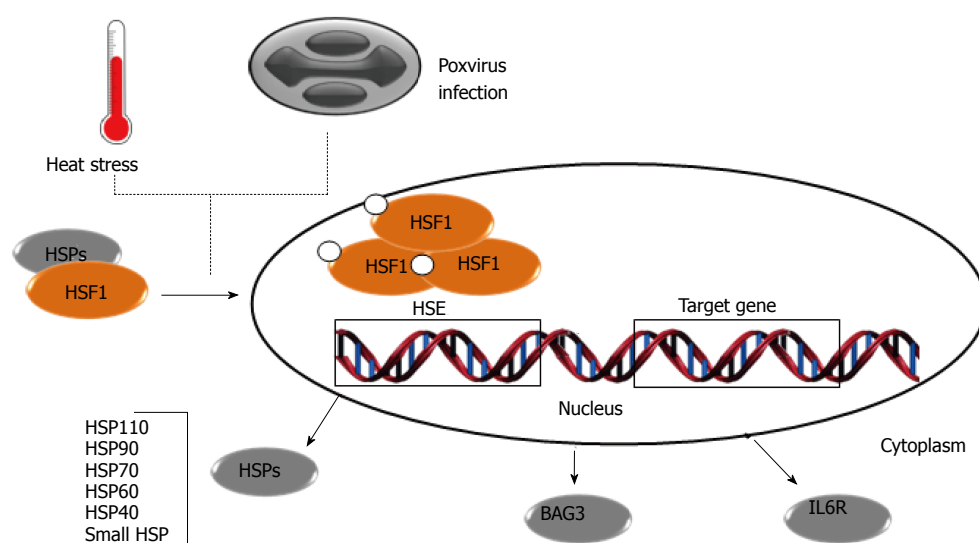
As soon as these viruses enter the host cell, they set in motion a number of biochemical strategies to usurp cellular resources. One such strategy is to hijack the host translation apparatus to selectively produce large quantities of viral proteins. To this end, poxviruses produce proteins that are able to cleave host messenger RNAs (mRNAs)<sup>[4,8-10]</sup> early in infection, shutting down the host protein synthesis almost completely during the first hours of the viral cycle<sup>[11]</sup>. Furthermore, viruses are devoid of molecular chaperones, such as heat shock proteins (HSPs) with few exceptions and rely almost completely on chaperones of the host to adequately process viral proteins,

avoiding misfolding or aggregation<sup>[12-14]</sup>. In parallel, viral double-stranded RNA intermediates, DNA and proteins are sensed by pattern recognition receptors in the cell, leading to the generation of innate immune responses potentially able to control the viral infection<sup>[15,16]</sup>.

All the above mentioned virus-driven interferences within the cell may lead to the transduction of cell stress signals and consequent cell stress responses. The cell may respond to stress in a variety of ways, including the activation of pathways that promote survival or the elimination of damaged cells through programmed cell death (apoptosis, necrosis and/or autophagy). There is a multitude of pathways that may be elicited upon different types of stress and the resulting signal transduction cascades are often shared by other cell processes, such as the activation of innate immunity, cell cycling and so on. Nevertheless, the most common stress responses include those elicited against heat shock, ER stress (the unfolded protein response, UPR), DNA damage, hypoxia and oxidative stress. Some of these responses may limit or inhibit viral replication and/or induce cell death and others can promote cell survival and restore homeostasis. To cope with stress responses, poxviruses have evolved complex molecular strategies to counteract innate host cell defense signaling pathways while facilitating biological events that promote adaptation and survival of the host cell, all essential to a productive infection. This review summarizes the main cellular stress responses used or subverted by poxviruses to ensure completion of viral life cycle.

## HEAT SHOCK RESPONSE

In the early 1960s, the discovery of the heat shock response (HSR) led to the elucidation of some aspects of the cell stress responses and the discovery of heat shock genes<sup>[17]</sup> and proteins (HSPs)<sup>[18,19]</sup>. Many HSPs are constitutively present in cells while some are expressed only after stress. HSPs and other molecular chaperones (*e.g.*, co-chaperones and folding enzymes) are active in a myriad of biological essential processes that include: (1) the normal folding of polypeptides; (2) assisting misfolded proteins to attain or regain their native states; (3) regulation of protein degradation; and (4) translocation of proteins across membranes to different cellular compartments<sup>[20,21]</sup>. Some of these proteins are conserved in all three superkingdoms and are encoded by genes that contain cis-acting regulatory sequences, termed heat shock elements (HSE), which are regulated by heat shock transcription factors (HSFs)<sup>[22,23]</sup>. Upon stress, one of the main regulators of the HSR, the HSF1, undergoes trimerization and subsequent translocation into the nucleus where these complexes bind to the HSE<sup>[23]</sup> (Figure 1). HSF1 is regulated by post-translational modifications such as phosphorylation, acetylation<sup>[24]</sup>, sumoylation<sup>[25,26]</sup> and interactions with other proteins. HSF1 is constitutively expressed and is neither a stress-inducible protein nor is its expression correlated with the



**Figure 1 Heat shock responses induced by poxviruses.** Under normal conditions, HSFs interact with HSPs or exist as a monomer in the cytosol. Upon exposure to stress conditions such as heat shock, oxidative stress or poxvirus infection, HSF1 undergoes post-translational modifications, such as phosphorylation, trimerizes and migrates to the nucleus. In the nucleus, HSF1 trimer binds to the HSE, leading to induction of all classes of HSPs and other chaperones. HSE: Heat shock elements; HSFs: Heat shock transcription factors; HSPs: Heat shock proteins.

expression of heat shock genes<sup>[27]</sup> (Figure 1).

Recent studies, using different genome-scale approaches to identify host proteins used by poxviruses during infection, revealed that HSF1 is a crucial transcription factor for virus replication and some targets of HSF1 are induced upon infection<sup>[28]</sup>. At the early stages of poxvirus infections, a decrease in HSF1 mRNA synthesis is observed; however, this does not seem to affect the protein levels as its half-life is quite long. As the viral lifecycle progresses, an increase in HSF1 mRNA levels can be detected, although this is not followed by augmentation in this protein contents within the cell<sup>[29]</sup>. Infections by some poxviruses result in the phosphorylation of HSF1 and its translocations to nucleus, where they bind to HSE<sup>[28,29]</sup>. Several HSF1-regulated genes are upregulated during infection, including genes coding for the molecular chaperones *BAG3*, *STIP1*, all classes of HSPs (HSP10, HSP20, HSP40, HSP60, HSP70, HSP90 and HSP105/110) and other important proteins like *IL6R*, which has a role in cell growth and differentiation<sup>[8,28,30]</sup> (Figure 1).

The first observation of the interaction between poxvirus and HSPs was made by Jindal *et al.*<sup>[10]</sup> (1992) who also showed that the infection led to a small increase in HSP90 and HSP60 mRNA contents and to a substantial increase in the HSP70 mRNA levels, suggesting that these proteins may play some role in viral protein folding. Opposed to this view, subsequent studies revealed that the overexpression of the 72 kDa HSP, the major inducible cytoplasmic HSP, did not affect virus replication<sup>[31,32]</sup>. Furthermore, during poxvirus infections, HSP70 accumulates predominantly in the nucleus where these proteins interact with poly (ADP-ribose) polymerase 1, PARP1 and XRCC1 and prevent single-stranded DNA

break (SSB)<sup>[29,33]</sup>. Globally, these observations suggest that HSP70s are important for cell survival and death prevention but may have a lesser impact in the proper folding of poxviral proteins.

So far, the most likely HSP to have a role in the poxvirus life cycle is HSP90. This chaperone is the most abundant HSP in unstressed cells and many of its targets are either kinases or transcription factors such as Akt and HSF1, respectively<sup>[34,35]</sup>. The inhibition of HSP90 function during infection by the use of geldanamycin, a drug that blocks the ATPase activity of that chaperone, impairs viral multiplication by delaying viral DNA replication and intermediate transcription, and also by reducing expression of late genes<sup>[36]</sup>.

It has been shown that HSP90 interacts directly with the 4a core protein (encoded by *A10L* orthologous genes), implicating this chaperone in conformational maturation of the poxvirus capsid. Nonetheless, HSP90 does not colocalize with capsid proteins at later stages of infection, suggesting a transient role for HSP90 in virion morphogenesis<sup>[36]</sup>. Other host chaperones (*e.g.*, cyclophilin A and Hsc71) are found to be associated with intracellular mature virions (IMV) but the importance of these proteins in such a context needs be further investigated<sup>[37,38]</sup>.

## UNFOLDED PROTEIN RESPONSE

The endoplasmic reticulum (ER) is a multifunctional organelle that controls several critical aspects of cellular processes: it ensures the correct structure of most proteins; plays a key role in the synthesis of lipids and sterols; and helps in the maintenance of intracellular calcium levels and many other functions<sup>[39]</sup>. The protein homeo-



It is known that XBP1 can be activated by TLR-2 and TL4-4 stimulation in an IRE1 dependent manner; also known is the fact that Vaccinia virus and other chor-

dopoxviruses are able to interfere with TLR signaling. Therefore, this seems to be a virus-driven indirect strategy to down-modulate XBP1 activation. Because XBP1 has been shown to be important for sustained production of cytokines by macrophages, it seems logical that poxvirus may interfere with XBP1 activation as a way to cope both with the host innate responses as well as with the ER stress.

Another component of the UPR, PERK (also known as eIF2 $\alpha$ K3) shares homology to the IRE1 structure and activation pathways but lacks the RNase domain of IRE1<sup>[63]</sup>. Like the IRE1 activation, the release of BiP from PERK triggers dimerization of the later and its transphosphorylation (Figure 2). The activated PERK dimer is capable of recognizing and phosphorylating the alpha subunit of the translation initiation factor eIF2 $\alpha$  at serine 51, reducing the translation of virus and cell mRNAs<sup>[64]</sup> (Figure 2). On the other hand, eIF2 $\alpha$  phosphorylation upregulates the translation of ATF4, which induces expression of CHOP, GADD34, ATF3<sup>[65-67]</sup> and other genes involved in processes that are usurped and modulated during poxvirus replication, including amino acids transport<sup>[11]</sup>, glutathione metabolism<sup>[68]</sup> and control of oxidative stress<sup>[69]</sup>. Not surprisingly, poxviruses encode proteins that mimic eIF2 $\alpha$  and act as a pseudosubstrate for PERK, consequently suppressing phosphorylation of eIF2 $\alpha$  and the shutoff of viral protein synthesis<sup>[70,71]</sup> (Figure 2).

## HOST TRANSLATIONAL SHUTOFF

Most viruses, as obligate intracellular parasites, lack most genes related to the transcriptional and translational machinery, including those coding for enzymes, transcriptional factors, ribosomal subunits, translation factors and transfer RNAs (tRNA). Poxviruses encode their own transcriptional machinery but, to ensure viral mRNA translation during productive infections, they must effectively govern the host translation apparatus while avoiding stress responses like the eIF2 $\alpha$  phosphorylation mediated translation shutoff.

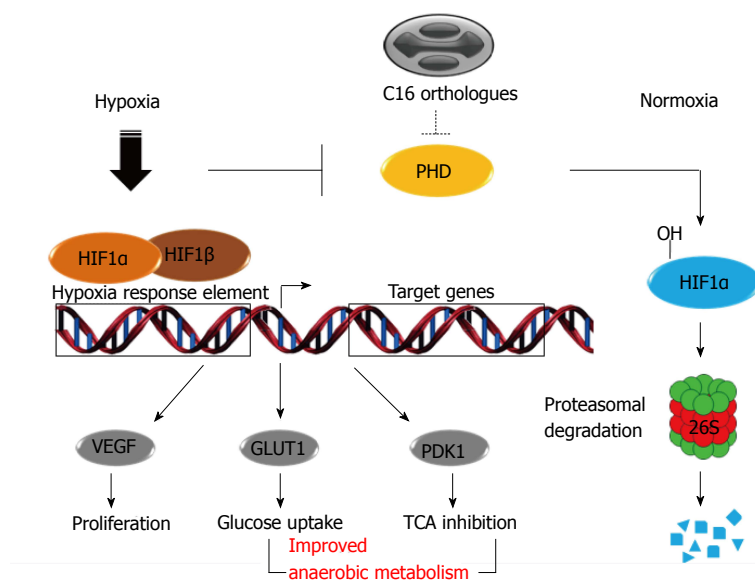
In addition to PERK, which is involved in responses to the proteostasis imbalance in the ER, three other stress-activated eIF2 $\alpha$  kinases are capable of inducing a broad range of responses designed to protect the cell. Protein kinase R (PKR), heme-regulated inhibitor (HRI) and general control nonderepressible 2 (GCN2) respond to dsRNA, oxidative stress and nutrient deprivation, respectively<sup>[72-74]</sup>. PKR (also known as eIF2 $\alpha$ K2) is activated in response to stress signals usually resulting from viral infections and, together with other sensing and responding pathways that lead to eIF2 $\alpha$  inactivation, is part of the so called integrated stress response (ISR). Poxviruses evolved non-redundant strategies to suppress activation of ISR and collectively inhibit the host translational shutoff response. The best characterized poxvirus' strategy to evade ISR is the expression of a pleiotropic viral protein, encoded by *E3L* orthologous genes, which is able

to bind dsRNA and inhibit PKR activation. Nonetheless, other viral proteins also play critical roles in this process, including those encoded by *K1L*, *C7L* and *CP77L* orthologues<sup>[75-77]</sup>. Poxviruses lacking *E3L* orthologous genes induce the formation of host-protein dense antiviral granules (AVGs) that suppress translation of viral but not stress-induced host mRNAs and thus inhibit poxvirus replication<sup>[78]</sup>.

ISR activation often promotes the formation of ribonucleoprotein aggregates called stress granules (SGs) at random sites throughout the cytoplasm. These SGs function as a protection zone for host RNAs where they can be stored when intracellular conditions are harmful<sup>[79]</sup>. SGs are distinct from AVGs in function and composition but share some components, like mRNA and RNA binding proteins [including Ras GTPase-activating protein-binding protein 1, Caprin-1, TIA1 and mRNA poly(A) binding protein, PABP] and other translation initiation components [including eIF3H and eIF4A/E/G (eIF4F complex) with the exception of 40S ribosomal subunits and eIF3B which only localize to SGs]<sup>[80,81]</sup>; both granules, nevertheless, are dependent on translation repression. In productive poxviral infections, some of these granule components (as well as eIF4E and eIF4G) are sequestered to viral factories where they assemble and form eIF4F complexes that act together with PABP to promote activation of mRNAs harboring 7-methyl GTP caps and poly(A) tails<sup>[82]</sup>. Poxvirus mRNAs are capped on their 5' ends by the action of a viral methyl transferase enzyme complex<sup>[83-85]</sup> and are also polyadenylated by a complex mechanism involving repetitive transcription of thymidylates in the sequence 3'-ATTTA-5' often present at the sites of transcriptional initiation<sup>[86,87]</sup>. By sequestering molecules that activate capped and polyadenylated mRNAs to the viral factories, poxviruses are able to vigorously boost the translation of their own mRNAs.

## HYPoxic RESPONSE

Molecular oxygen (O<sub>2</sub>) is an essential element to aerobic organisms that serves as a key substrate in cellular metabolism and bioenergetics. Hypoxic stress response is the process by which cells react and adapt to an insufficient O<sub>2</sub> availability (or hypoxia)<sup>[88]</sup>. During hypoxic conditions, cells activate a number of adaptive responses to match O<sub>2</sub> supply with metabolic, bioenergetic and redox demands. The hypoxia-inducible factor-1 (HIF-1) is the key regulator of the cell resilience in response to O<sub>2</sub> deprivation and it is regulated by prolyl hydroxylase domain-containing enzymes (PHDs)<sup>[89,90]</sup>. HIFs are obligate heterodimers, consisting of an O<sub>2</sub>-destructible  $\alpha$ -subunit and O<sub>2</sub>-indestructible  $\beta$  subunit, and under physiologically normal O<sub>2</sub> levels (normoxia), PHDs mediate hydroxylation of proline residues in the HIF $\alpha$  subunit, triggering their recognition and labeling by E3 ubiquitin ligases, which leads in turn to their proteasomal degradation<sup>[91,92]</sup>. PHD activities are regulated by O<sub>2</sub> availability and by cellular metabolites such as tricarboxylic acid cycle



**Figure 3 Hypoxic responses in poxviruses infected cells.** Under normal O<sub>2</sub> disponibility (normoxia), HIF1α is hydroxylated on proline residues by PHDs. After that, HIF1α is recognized and ubiquitinated by E3 ubiquitin ligase and undergoes proteasomal degradation. Upon an insufficient O<sub>2</sub> availability (hypoxia), PHD become inactive and HIF1α forms heterodimers with HIF1β and triggers expression of regulators of TCA, cell proliferation and glucose metabolism. Poxviruses C16L orthologous genes code for proteins that inhibit PHD activities and result in expression of hypoxia target genes under normoxia conditions. HIF1: Hypoxia-inducible factor-1; PHD: Prolyl-hydroxylase domain-containing enzyme; TCA: Tricarboxylic acid cycle; VEGF: Endothelial growth factor; GLUT1: Glucose transporter-1; PDK1: Pyruvate dehydrogenase kinase-1.

(TCA) intermediates<sup>[93]</sup>. Due to the lack of sufficient O<sub>2</sub> upon hypoxia, PHDs become inactive and HIFα is consequently stabilized, causing the HIFs translocation to the nucleus where they bind to hypoxic responsive elements present in genes, such as *HSPA5*, *Fos*, *CXCR*, among other genes related to signal transduction, cell metabolism, apoptosis, *etc*<sup>[94-96]</sup> (Figure 3).

There are three PHD isoforms but PHD2 is believed to be the primary regulator of the HIF transcription factors<sup>[88]</sup>. The Vaccinia virus C16 protein is non-essential for virus replication but seems to play an important role in the down-modulation of the host immune responses<sup>[97]</sup>. Further studies showed that this protein can inhibit HIF1α hydroxylation through direct interaction with the PHD2 enzyme even when ectopically expressed<sup>[98]</sup>. Consequently, HIF1α is not ubiquitinated and degraded by proteasome, leading to the stabilization of this factor and up-regulation of HIF-responsive genes [endothelial growth factor (*VEGF*), glucose transporter-1 (*GLUT1*) and pyruvate dehydrogenase kinase-1 (*PDK1*)], improving cell metabolism and creating conditions that favor virus replication (Figure 3).

## OXIDATIVE STRESS RESPONSE

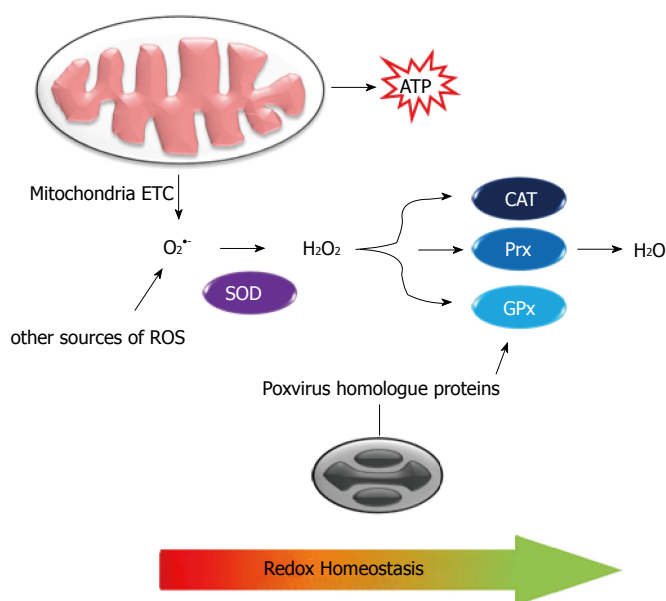
Poxviruses exploit the *de novo* fatty acid biosynthesis in the cell and especially the production of palmitates. These molecules undergo β-oxidation in mitochondria and, together with the glutamine catabolism, generate acetyl-CoA and α-ketoglutarate, respectively. Both molecules enter in the TCA cycle and are used as major energy sources instead of glucose in infected cells<sup>[68,99,100]</sup>. In this metabolic pathway, O<sub>2</sub> plays a pivotal role as the final electron acceptor of oxidative phosphorylation coupled to the electron transfer chain, resulting in the production of water (H<sub>2</sub>O), but also superoxide anion (O<sub>2</sub><sup>•-</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), as well as other reactive oxygen species (ROS)<sup>[101,102]</sup> (Figure 4). ROS can significantly damage cell structures, causing lipoperoxidation,

protein denaturation and DNA degradation; but on the other hand, ROS acts as a second messenger in mediating inflammation, stimulating cell proliferation and regulating apoptosis to maintain cell homeostasis<sup>[103]</sup>. Due to their cytotoxicity activity, cellular ROS levels are tightly limited by multiple detoxification processes such as antioxidant enzymes and vitamins whose functions are collectively appointed as an oxidative stress response<sup>[102]</sup>.

ROS are usually controlled by antioxidant enzymes such as cooper/zinc-dependent superoxide dismutase (SOD) (cytoplasm), manganese-dependent SOD (mitochondria) and extracellular-SOD (also utilizes Cu/Zn as cofactor), which dismutate O<sub>2</sub><sup>•-</sup> into H<sub>2</sub>O<sub>2</sub>. Hydrogen peroxides are in turn decomposed by catalase (CAT) and peroxidases such as glutathione peroxidase (GPx)<sup>[104]</sup> (Figure 4).

It has been shown that Myxoma virus and Shope fibroma virus increase intracellular ROS accumulation to promote growth of infected cells and immune evasion. This is achieved *via* inhibition of Cu/Zn-SOD1 activity through the expression of catalytically inactive homologs of cellular SOD1 that cannot bind Cu, which is essential for dismutase activity but retains the Zn-binding properties and, similarly to their cellular homologs, forms stable heterodimeric complexes with cellular Cu-dependent chaperones that are essential for SOD1 function<sup>[69,105]</sup> (Figure 4). It is likely that other poxviruses cause a similar effect during their multiplication cycle as some encode SOD-1 like genes; one such example is the A45R SOD-1-like gene from Vaccinia virus. Besides the SOD1 homologues, another known poxvirus gene product that can alter the redox state in infected cells is the *Molluscum contagiosum* virus MC066L gene product, which is homologous to the human GPx<sup>[12]</sup>, an enzyme able to protect cells from the proapoptotic peroxides generated by ultraviolet (UV) light<sup>[106]</sup> (Figure 4).

Cellular peroxiredoxins and thioredoxins, among other host proteins that are not essential to the cellular redox state (*e.g.*, 60S ribosomal proteins, HGM1 and



**Figure 4 Role of poxvirus proteins in cell redox homeostasis.**

ROS are produced during physiological and stress conditions, for instance, during energetic metabolism in the mitochondria, and are detoxified by cellular enzymes (SOD, CAT, Prx, GPx) into water and oxygen. Poxviruses code proteins with homology to SOD, inhibiting the conversion of superoxide into hydrogen peroxide. Furthermore, MC066L gene product is homologous to the human GPx and can protect host cells of peroxide accumulation. ROS: Reactive oxygen species; SOD: Superoxide dismutase; CAT: Catalase; Prx: Peroxiredoxin; GPx: Glutathione peroxidase.

Rab10), can be detected in IMV particles. It has been speculated that those redox regulation proteins may play some role in virion maturation<sup>[38]</sup>. Indeed, redox conditions seem to be so important for poxviruses that many of them encode their own redox machinery in order to mediate disulfide bond formation in newly made viral proteins<sup>[107-109]</sup>.

## DNA DAMAGE RESPONSE

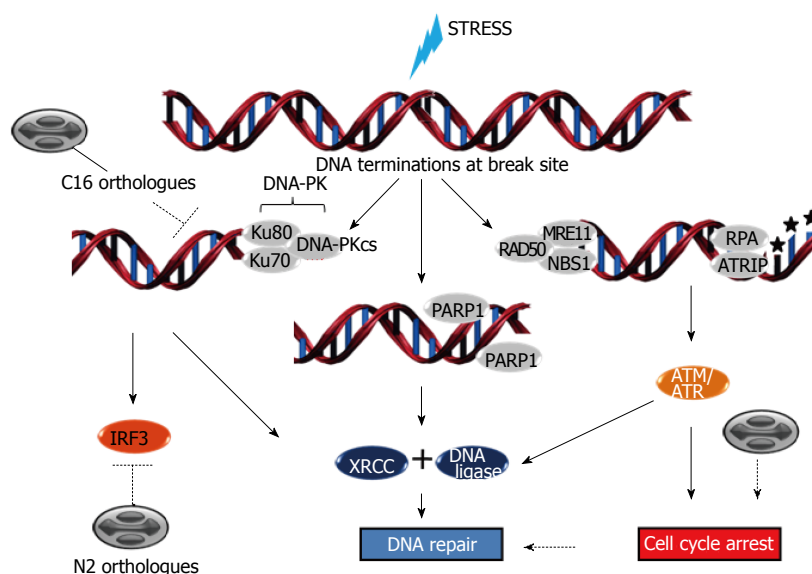
Several reports correlate stressful conditions with DNA damage responses (DDR). Hypoxia, ROS accumulation, ER stress, heat shock and mainly UV light exposure are conditions that either result or are resultant from DNA damage and whose sensing by the cell might contribute to the global stress adaptation response fostering cell resilience<sup>[96,110-114]</sup>. DDR events operate in diverse biological settings such as telomere homeostasis and generation of immune-receptor diversity<sup>[115]</sup> and include cell cycle checkpoint control, transcription, activation of DNA repair pathways, senescence and/or apoptosis. DNA damage can be subdivided into a few major types, including DNA double-strand breaks (DSB), DNA nucleotide adduct formation and base modification, DNA base pairing mismatches and single-strand breaks (SSB) which are caused by exposure to chemotherapeutic agents or environmental genotoxic agents such as polycyclic hydrocarbons and UV radiation. Accordingly, the major classes of DNA repair are DNA dsb repair by homologous recombination (HR) or nonhomologous end-joining (NHEJ), nucleotide excision repair, base-excision repair (BER), the Fanconi anemia/BRCA pathway and nucleotide mismatch repair<sup>[116]</sup>. The central sensor proteins in the DDR signal transduction cascade (ataxia telangiectasia mutated-ATM, ataxia telangiectasia and Rad3 related-ATR, DNA-dependent protein kinase-DNA-PKcs) belong to the phosphoinositide-3-kinase-related kinase (PIKK) family,

with the exception of proteins from the PARP family which also respond to DNA lesions<sup>[117]</sup> (Figure 5).

ATM is recruited by the MRE-11-Rad50-NBS1 (MRN) complex to sites of DSBs and phosphorylates downstream substrates such as checkpoint kinase 2 (Chk2) which, subsequently phosphorylates p53 that in turn signals through p21 to slow the cycling of cells in order to facilitate DNA repair<sup>[118]</sup> (Figure 5). If the damage is too severe to be repaired, the cascade leads to death signalization through pro-apoptotic proteins. In the case of SSBs, ATR is recruited to damage sites in association with ATR-interacting protein by replication protein A (RPA). Once activated, these complexes phosphorylate Chk1 which, in turn, phosphorylates and inhibits cdc25c to mediate G<sub>2</sub>/M arrest (or, alternatively, phosphorylates cdc25a to promote S-phase arrest). Most ATR substrates can also be phosphorylated by ATM and the major functions of ATR and ATM in cell cycle control are overlapping but non-redundant<sup>[119,120]</sup>. These signaling cascades appear to be the major repair pathways influenced by poxvirus infections (Figure 5) as they favor cell cycle progression to G<sub>1</sub>, S and G<sub>2</sub> phases but arrest cells in the G<sub>2</sub> phase. Indeed, there is a preferential accumulation of poxvirus infected cells in G<sub>2</sub>/M phases concurrent with a decrease in the number of cells in the G<sub>0</sub>/G<sub>1</sub> ones<sup>[121,122]</sup>.

The NHEJ repair pathway is initiated by association of Ku70/80 proteins to the DNA ends and the subsequent recruitment of the DNA-dependent protein kinase catalytic subunit (DNA-PKcs)<sup>[123,124]</sup>. These proteins localize both in the nucleus and the cytoplasm and are key factors in the immune response signaling, acting as viral dsDNA sensors leading to the induction of interferon regulatory factor 3 (IRF3) in a TANK-binding kinase 1-dependent manner<sup>[125]</sup>. Counteracting this immune signaling, the Vaccinia virus produces the C16 protein early in infection, which can bind to Ku70 blocking DNA-PK recruitment to DNA and the N2 protein, a virulence fac-





**Figure 5 DNA damage responses and poxvirus infections.** DNA breaks may be caused by many different sources. At sites of DNA double strand breaks (DSBs), DNA-PK is recruited by Ku proteins and induces DNA repair through XRCC4 and DNA ligase 4; DSBs also lead to the activation of the major interferon regulatory factor, IRF3. Upon DSBs occurrence, ataxia telangiectasia mutated (ATM) is recruited by the MRE-11-Rad50-NBS1 (MRN) complex to sites of broken DNA where they induce repair by XRCC2/3 and DNA ligase 1. ATM also controls cell cycle arrest which facilitates proper function of the DNA repair mechanisms. Upon single strand breaks, ataxia telangiectasia and Rad3 related (ATR) or poly (ADP-ribose) polymerase 1 (PARP1) are recruited to lesions sites and are activated, resulting in phosphorylation of downstream substrates, control of cell cycle arrest and/or repair of DNA lesions by XRCC1 and DNA ligase 3. Poxvirus infections affect cell cycle progression arresting cells in G<sub>2</sub> phase. They also encode C16 orthologues that bind to Ku70, blocking DNA-PK recruitment to broken DNA sites, and N2 orthologues, that inhibit IRF3-dependent innate immune responses. DNA-PK: DNA-dependent protein kinase; DNA-PKcs: DNA-dependent protein kinase catalytic subunit; RPA: Replication protein A.

tor that presents with the ability to inhibit IRF3-dependent innate immune responses<sup>[126,127]</sup> (Figure 5).

Poxviruses exploit their own replication machinery in order to repair eventual lesions at the viral DNA<sup>[3]</sup>, mainly through the action of virally encoded uracil DNA glycosylases (coded by *D4R* orthologous genes), which initiate BER by hydrolyzing the glycosylic bond linking uracil to a deoxyribose sugar, and also through the repair of nicked duplex DNA substrate by a viral DNA ligase, a product of the *A50R* ORF present in some chordopoxviruses<sup>[128-131]</sup>. Furthermore, the viral DNA polymerase (coded by *E9L* gene orthologues) which possess 3' - 5' proofreading exonuclease activity and the *G5R* gene product which belongs to FEN1-like nucleases appear to conjunctly play important roles in viral DNA recombination through HR<sup>[132-136]</sup>. The cellular DNA ligase I can compensate an eventual absence of the viral DNA ligase and is recruited from the nucleus to the cytoplasmic viral factories. However, in the absence of a G5 protein, the viral DNA is fragmented and cannot be packaged<sup>[136,137]</sup>.

## MISCELLANEOUS CELL SIGNALING

### PI3K/Akt signaling pathway

The phosphoinositide-3-kinase (PI3K) family of enzymes is grouped into three classes of proteins. PI3K is activated by G protein-coupled receptors and tyrosine kinase receptors to drive phosphorylation of inositol lipids at the 3' position of the inositol ring, generating lipid second messengers [3-phosphoinositides PI(3)P, PI(3,4)P<sub>2</sub> and

PI(3,4,5)P<sub>3</sub>]<sup>[138,139]</sup>. Class IA PI3K proteins were shown to play an important role in poxvirus infections, promoting Akt phosphorylation and downstream events leading to the suppression of apoptosis, cell growth, survival and proliferation<sup>[140,141]</sup>. The PI3K/Akt pathway seems to be a determinant for the replicative success of Vaccinia virus and Cowpox virus, as well as for the host cell survival during infection, as the pharmacological impairment of the pathway components leads to diminished virus multiplication and apoptosis<sup>[141]</sup>.

### MAPK signaling pathway

Stress conditions (osmotic stress, ER stress, among others), growth factors and/or cytokines stimulate the activation of mitogen-activated protein kinases (MAPK)<sup>[142,143]</sup>. The MAPK family consists of a series of at least three main kinases active through distinct pathways: the extracellular signal-regulated protein kinases (ERKs), the c-Jun N-terminal kinases (JNKs) and the p38 family of kinases. These MAPK enzymes are activated by post-translational modifications induced by specific kinases, named MAPK kinases (MAP2K), which are activated by upstream MAPKK kinase (MAP3K) [Raf, MAPK/ERK kinase (MEKKs) and apoptosis signal-regulating kinase (ASK)]<sup>[144]</sup> and which in turn respond either to external stimuli sensed by receptors on the cell surface or through interactions with GTP-binding proteins, among other kinases. Poxviruses have been shown to trigger mitogenic signals at early stages of infection, resulting in the expression of *egr-1* and other genes, such as the proto-

oncogene c-fos, through the activation of ERK1/2. This process is essential for multiplication of some members of this viral family as blocking of those kinases hampers normal virus multiplication<sup>[145-147]</sup>. Additionally, the JNK pathway is also important for normal virus morphogenesis and accumulation of enveloped infectious forms<sup>[148]</sup> as blocking of the pathway influences cell-to-cell virus spread.

## CONCLUSION

The activation of cellular stress responses in infected cells is a complex process that promotes simultaneously both cell resilience and death mechanisms upon a viral infection. In order to achieve replicative success in such conditions, poxviruses must subvert these cell responses to their own benefit. Members of the Poxviridae family are fully geared up to interfere with and manipulate cell fate in a way that very few other animal viruses do. They have unique abilities to turn off and/or combat negative effects of stress responses while still fomenting mechanisms to support the completion of its life cycle. Overall, poxviruses modulate the activation of a network of protein kinases (PI3K, PIKKs, MAPKs) and other enzymatic post-translational modifiers, such as the ubiquitin ligases and proteins involved in cell reprogramming (including ATFs, HSFs, XBP1, HIFs), while selectively inhibiting the activation or expression of host proteins (DNA-PK, IRF3, PHDs, PKR, PERK among others). In parallel, they are able affect the cell metabolism and redox state, maintaining proteostasis (through HSPs and other hosts and viral chaperones) and controlling cell cycle and proliferation in order to establish a proper cell environment for virus replication. Many of these strategies are highly conserved among different poxviruses, while a few others are species-specific<sup>[149]</sup>. The evidence of horizontal gene transfer from host to virus, coupled with the proposed model of poxvirus genome evolution based on a simple mechanism of recombination-driven genomic expansions and contractions (which facilitates the rapid evolution of virus populations with otherwise low mutation rates), sheds light on how these viruses acquired this impressive number of strategies to wisely control their replication niche<sup>[150-152]</sup>.

Over 50 years after the discovery of HSR by Ferruccio Ritossa<sup>[153]</sup>, the cellular stress response knowledge is still growing (including specific organelle stress such as mitochondrial or peroxisomal UPR, Golgi stress response and so on) and the understanding of mechanisms by which poxviruses and other complex viruses interfere with stress responses can further illuminate the web of pathways regulating cell homeostasis, as well as how viruses intertwine their own biochemical needs into this intricate scenario.

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## DNA methylation in liver diseases

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### Abstract

Recently, growing evidences show that the combination of epigenetic and genetic abnormalities contribute together to the development of liver diseases. DNA methylation is a very important epigenetic mechanism in human beings. It refers to addition of the methyl groups to DNA and mainly occurs at cytosine adjacent to guanine. DNA methylation is prevalent across human genome and is essential for the normal human development, while its dysfunction is associated with many human diseases. A deep understanding of DNA methylation may provide us deep insight into the origination of liver diseases. Also, it may provide us new tools for diseases diagnosis and prognosis prediction. This review summarized recent progress of DNA methylation study and provided an overview of DNA methylation and liver diseases. Meanwhile, the association between DNA methylation and liver diseases including hepatocellular carcinoma, liver fibrosis, nonalcoholic steatohepatitis and liver failure were extensively discussed. Finally, we discussed the potential of DNA methylation

therapeutics for liver diseases and the value of DNA methylation as biomarkers for liver diseases diagnosis and prognosis prediction. This review aimed to provide the emerging DNA methylation information about liver diseases. It might provide essential information for managing and care of these patients.

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**Key words:** DNA methylation; Liver diseases; Hepatocellular carcinoma; Liver fibrosis; Nonalcoholic steatohepatitis; Liver failure

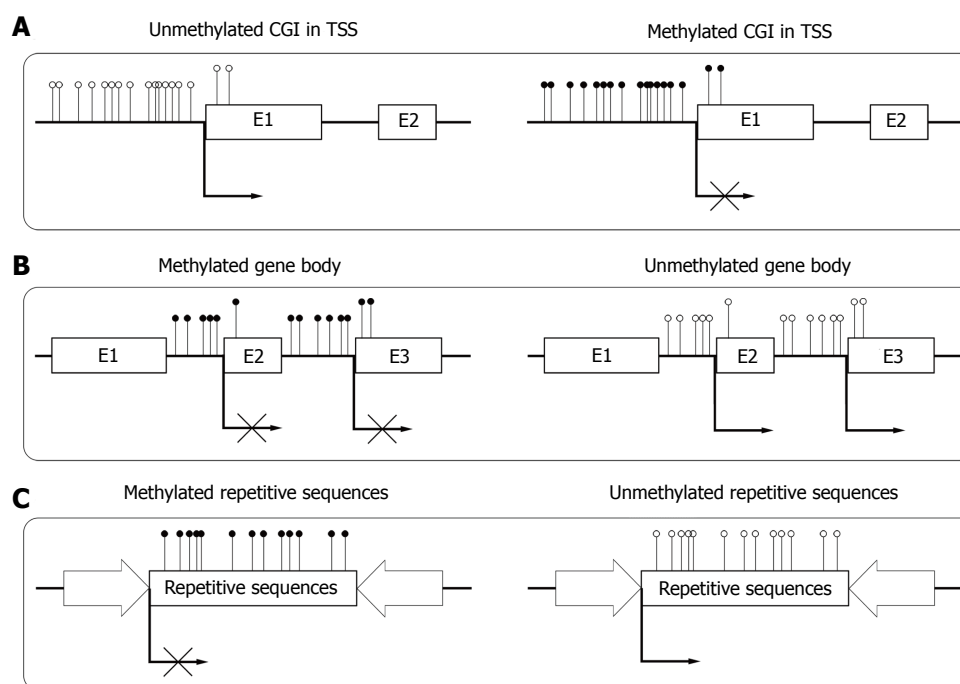
**Core tip:** This review summarized recent progress of DNA methylation study and provided an overview of DNA methylation and liver diseases. The association between DNA methylation and liver diseases including hepatocellular carcinoma, liver fibrosis, nonalcoholic steatohepatitis or liver failure were extensively discussed. We also discussed the potential of DNA methylation as biomarkers and therapeutic targets for liver diseases. This review aimed to provide the emerging DNA methylation information about liver diseases. It might provide essential information for managing and care of these patients.

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### INTRODUCTION

Because of the high prevalence, liver diseases have been studied systematically during the past few decades. Many studies focus on genetic defects<sup>[1]</sup> and genome-wide association studies do provide us great information about the pathogenesis of liver diseases<sup>[2]</sup>. However, many questions which cannot be totally illustrated by genetic mechanism still exist, which lead researchers to initiate





**Figure 1** DNA methylation pattern in different parts of the genomes. The normal conditions are presented in the left column and aberrant conditions are shown on the right. The black dots represent methylated CpG sites and the white circles represent unmethylated CpG sites. A: In normal cells, CpG islands (CGI) in transcriptional start site (TSS) usually remain unmethylated, allowing transcription. Aberrant methylation often links to long-term stabilization of transcriptional silencing and loss of gene function both physically and pathologically; B: In normal cells, gene bodies are CpG-poor and extensively methylated, increasing elongation efficacy. Aberrant demethylation of gene bodies may facilitates spurious initiations of transcription; C: In normal cells, repetitive sequences of genome are highly methylated, preventing chromosomal instability or gene disruption. Aberrant demethylation of repetitive sequences may reactivate endoparasitic sequences.

the study of epigenetic variation. Recent studies showed that the combination of genetic and epigenetic variants contributed together to the susceptibility and progression of liver diseases<sup>[3-5]</sup>. Epigenetics refers to the heritable changes of gene expression without changes in gene sequence<sup>[6]</sup>. DNA methylation is a very important epigenetic mechanism in human and distribute widely across human genome. It is of crucial important for normal development, genomic imprinting as well as inactivation of X-chromosome<sup>[7-9]</sup>. Meanwhile, aberrant DNA methylation usually associates with many human diseases<sup>[10]</sup>. The goal of this article is to review the studies associated with DNA methylation and liver diseases. Finally, we look into the future prospect that DNA methylation may bring to the detection and treatment of liver diseases.

## DNA METHYLATION AND ITS MECHANISM

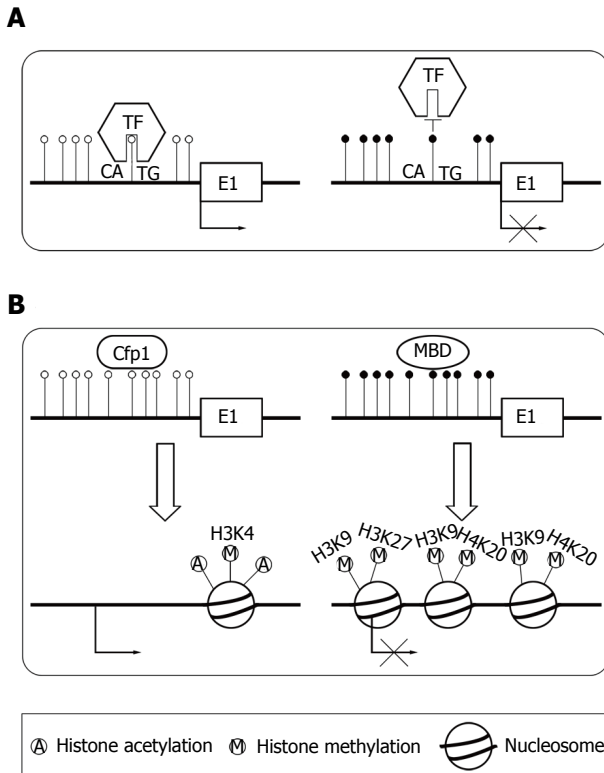
DNA methylation which refers to addition of the methyl groups to DNA is firstly introduced in 1970s<sup>[11,12]</sup>. In invertebrates and fungi, DNA methylation only presents in small proportion of genome and varies among different clades<sup>[13,14]</sup>. In vertebrate genome, it presents in almost everywhere across the genome. Mainly, DNA methylation occurs at cytosine adjacent to guanine (CpG dinucleotides)<sup>[15]</sup>. In human genome, The CpG dinucleotides are very rare (approximately 1%). They are nonuniformly distributed and tend to cluster together to form CpG island

(CGI). CGI refers to a 200-bp region in DNA which is characterized by high G+C content (more than 50%) and high observed CpG/expected CpG ratio (at least 0.6)<sup>[16]</sup>. Previous studies showed that CGIs existed in more than half of the genes in vertebrate genomes. Until now, the exact role of gene methylation in gene regulation remains largely unclear<sup>[17]</sup>.

### DNA methylation in transcriptional start sites

Until now, most of the studies on DNA methylation focus on CGIs in the transcriptional start sites (TSSs) of genes. In human genome, about 60% of gene TSSs contain CGIs and usually remain unmethylated in normal cells. Methylation of these CGIs often result in long-term stabilization of transcriptional silencing and loss of gene function both physically and pathologically<sup>[18]</sup> (Figure 1A). CpG island shore is defined as lower CpG density region which is close (approximately 2 kb) to the CGI. Recent studies show that most tissue specific methylation occurs at CpG island shores<sup>[19,20]</sup>. Aberrant DNA methylation at CpG island shores correlate even more strongly with gene expression than CGI<sup>[21]</sup>.

There are about 40% of human genes which do not contain bona fide CGI at their TSSs<sup>[16]</sup>. Compared with genes that contained CGIs, the role of methylation in genes without CGIs at the TSSs has not been well demonstrated. More studies still need to be performed on genes without CGIs. Studies revealed that maspin gene had a promoter that did not reach the criteria for CGI



**Figure 2** Transcriptional suppression mechanisms of DNA methylation in TSSs. The normal conditions are presented in the left column and aberrant conditions are shown on the right. The black dots represent methylated CpG sites and the white circles represent unmethylated CpG sites. A: In normal cells, transcription factors (TF) bind to unmethylated binding site, allowing transcription. Aberrant methylated binding site prevent TF binding to its normal sites; B: In normal cells, unmethylated CpG island can recruit CpG binding proteins (Cfp1) and trigger histone modifications characterized by high levels of acetylation and trimethylated H3K4, H3K36 and H3K79. Finally, it forms a structure suitable for transcription. Aberrant methylated recruit methyl-CpG-binding domain (MBD) proteins and trigger histone modifications characterized by high levels of H3K9, H3K27 and H4K20 methylation and low levels of acetylation. It represses the transcriptional permissiveness of chromatin and results in gene silencing.

and hypermethylation of this promoter was strongly correlated with its tissue specific expression<sup>[22]</sup>. However, *MAGE* gene was found to be unregulated by methylation in the promoters which do not satisfy CGIs.

There are two primary means by which DNA methylation in TSSs repress transcription. The transcription factors<sup>[23]</sup> control gene expression level. DNA methylation can directly preclude the transcription factors binding to its normal sites<sup>[24,25]</sup> (Figure 2A). For example, transcription factor YY1 which is essential for the imprinting of *Peg3* gene can bind to PEG3-DMR sequence in the first intron<sup>[24]</sup>. *In vivo*, the methylation of PEG3-DMR sequence precludes the binding of YY1, which result in the repression of maternal allele. In paternal allele, YY1 can effectively bind to the unmethylated PEG3-DMR sequence. Alternatively, DNA methylation can recruit specific proteins and induce a repressive chromatin structure<sup>[9]</sup> (Figure 2B). In normal condition, unmethylated CGIs can recruit CpG binding proteins, which form a structure suitable for transcription<sup>[26]</sup>. When CGIs are methylated, they can recruit methyl-CpG-binding domain

(MBD) proteins<sup>[14,27]</sup>. Then, MBD proteins could recruit the histone modifying as well as chromatin remodeling complex to the methylated positions, which result in transcriptional silencing by repressing the transcriptional permissiveness of chromatin.

### DNA methylation in gene bodies

Although CGIs also exist within gene bodies<sup>[28]</sup>, most gene bodies are CpG-poor and extensively methylated. Studies showed that high level of gene body methylation was positively correlated with transcription, which meant it might associate with gene activation<sup>[29,30]</sup>. Zilberman *et al.*<sup>[31]</sup> found that the methylation of gene body could increase elongation efficiency and prevent spurious initiations of transcription (Figure 1B). Shukla *et al.*<sup>[32]</sup> illustrated that methylation between exons and introns was involved in regulating splicing<sup>[33]</sup>. Other studies reported that the methylation in gene body could be an important mechanism for managing promoter usage<sup>[34]</sup>. The high methylation level in gene body was essential for the elongation of a transcript.

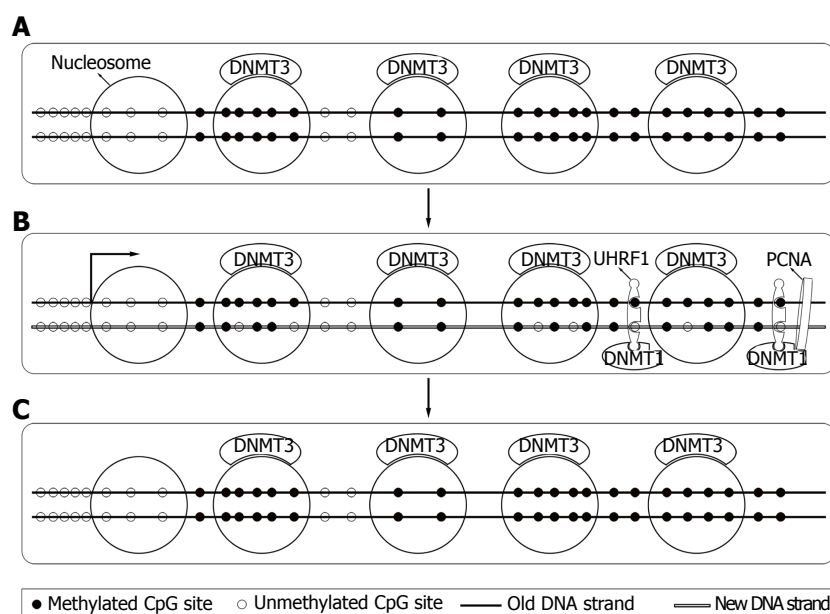
### DNA methylation in repetitive sequence

Repetitive elements comprise up to 45% of human genome<sup>[35]</sup>, which mainly consist of interspersed repeats and tandem repeats. In normal somatic cells, repetitive sequences of genome are highly methylated. The deeply methylated condition is essential for the stability of chromosome and normal gene expression<sup>[36]</sup> (Figure 1C). Demethylation of repetitive sequences in genome may result in different kinds of diseases<sup>[37,38]</sup>.

### The inheritance of DNA methylation

DNA methylation is an important way to store hereditary information. Although it does not change gene sequence, it can propagate the methylation mark during cell divisions<sup>[39]</sup>. The DNA methylation inheritance process is catalyzed by DNA methyltransferase (DNMT) enzyme family. Manly, there are five members in DNMT enzyme family, DNMT1, DNMT2, DNMT3a, DNMT3b and DNMT3L. DNMT1, DNMT3a, DNMT3b serve as methyltransferase. Each of the three DNMTs is essential for normal human development<sup>[7,40]</sup>. Studies revealed that loss of methylation resulted from the inactivation of DNMTs could result in apoptosis of somatic cell<sup>[41]</sup> and cancer cells<sup>[42]</sup>. However, it showed that DNMTs were not essential for the survival of embryonic stem cells<sup>[43]</sup>.

Bestor *et al.*<sup>[44]</sup> firstly cloned DNMT1 in 1988 from mouse cells. Later studies revealed that DNMT1 expressed mostly at S phase of cell cycle<sup>[45]</sup> and mainly acted as maintenance DNMT. Interacting with the DNA polymerase processing factor proliferating cell nuclear antigen and ubiquitin-like plant homeodomain and RING finger domain containing protein 1 (UHRF1), DNMT1 methylated the hemimethylated sites during DNA semi-conserved replication<sup>[46,47]</sup>. Soon after replication, DNMT3a and DNMT3b bound to methylated DNA and corrected methylation sites missed by DNMT1 and completed the



**Figure 3 The maintenance of DNA methylation pattern.** A: In somatic cells, DNA methyltransferase (DNMT) 3 (DNMT 3a and DNMT 3b) are bound to nucleosomes containing methylated DNA; B: During DNA semi-conservative replication, DNMT1 interact with the DNA polymerase processing factor proliferating cell nuclear antigen (PCNA) and ubiquitin-like protein 1 (UHRF1) and methylate the hemimethylated sites; C: Soon after DNA semi-conservative replication, DNMT3 correct methylation sites missed by DNMT1 and complete the process.

process<sup>[48,49]</sup> (Figure 3). DNMT1 was essential for both normal somatic cells and cancer cells and a knockout of DNMT1 could cause their death<sup>[41,42]</sup>.

After the cloning of DNMT1, studies found that embryonic stem cells could still methylate retroviral DNA de novo even without DNMT1<sup>[50]</sup>. DNMT3a and DNMT3b were found in later studies<sup>[40]</sup>. They were regarded as de novo DNMT and functioned to set up normal methylation pattern during embryonic development. They were abundant in embryonic stem cell and less expressed in differentiated cells<sup>[51]</sup>. Other DNMTs like DNMT3L possessed no methylation catalytical activities. But Bourc'h *et al.*<sup>[52]</sup> found that DNMT3L was crucial for establishment of maternal genomic imprinting.

## DNA METHYLATION AND HEPATOCELLULAR CARCINOMA

In hepatocellular carcinoma (HCC), DNA methylation is characterized by a genome wide hypomethylation and a site specific hypermethylation<sup>[53]</sup>. Until now, many studies for presenting the DNA methylation patterns in HCC have been published.

### Hypomethylation

Compared with normal liver tissue, DNA methylation in HCC is characterized by global hypomethylation. The hypomethylation of intergenic areas, repetitive DNA sequences<sup>[54]</sup>, introns<sup>[55]</sup> and promoter CGI of specific oncogene<sup>[56]</sup> are responsible for the global hypomethylation. Global hypomethylation mainly result in chromosomal instability, loss of genomic imprinting<sup>[57,58]</sup> and reactivation of transposable elements, which may contribute to the development of cancer.

Previous studies revealed that the demethylation of chromosome 1 heterochromatin DNA was associated with the q-arm copy gain<sup>[59]</sup> in HCC. Also, a number

of hypomethylated tumor-promoting genes, including HPA<sup>[60]</sup>, MAT2A<sup>[61]</sup>, VIM<sup>[62]</sup> and SNCG<sup>[63]</sup> have been identified in primary human HCC.

### Hypermethylation

In tumor suppressor gene, the hypermethylation of CGIs in TSSs result in the loss of gene function, which is crucial for the origin of cancer<sup>[64]</sup>. The inactivation of tumor suppressor genes caused by hypermethylation of CGI in TSS exist in almost every type of human cancers<sup>[65]</sup>. Hypermethylation may affect the process of cell cycle regulation, DNA repair, angiogenesis, programmed cell death and tumor cell invasion. The genes silenced by hypermethylation in human cancers are often those who are essential for the maintenance of stem cell characteristics and/or the maturation of adult cells during cell renewal<sup>[65,66]</sup>. Silencing of these genes may result in the initiation of tumors through distribution of abnormal stem cells and/or abnormal of normal cell differentiation.

Until now, many tumor suppressor genes have been identified to be hypermethylated in HCC. Table 1 presents a group of frequently methylated genes in HCC.

## DNA METHYLATION AND LIVER FIBROSIS

In liver fibrosis, aberrant DNA methylation has been studied for a few years. Until now, a number of aberrantly methylated genes have already been recognized. Through direct or indirect examination methods (treated with demethylating agents such as 5-aza-2'-deoxycytidine), these genes were identified to be aberrantly methylated. In activated hepatic stellate cell (HSC), transcriptional repression of some genes was indentified to be due to promoter hypermethylation of them.

Until now, genome-wide studies of DNA methylation associated with HSC activation were limited. Aberrant

**Table 1** A selection of methylated genes in hepatocellular carcinoma

Gene	Location	Function	Methylation frequency	Ref.
<i>GSTP1</i>	11q13.2	Detoxification	41%-58%	[85-87]
<i>SOCS1</i>	16p13.13	Cytokine inhibitor	60%	[88]
<i>RASSF1A</i>	3p21.3	Apoptosis	54%-95%	[89,90]
<i>E-Cadherin</i>	16q22.1	Cell adhesion	33%-67%	[91,92]
<i>APC</i>	5q22.2	Signal transduction	46%	[93]
<i>p16</i>	9q21.3	CDK inhibitor	17%-83%	[94,95]
<i>SFRP1</i>	8p11.21	Signal transduction	59.50%	[96]
<i>WIF-1</i>	12q14.3	Signal transduction	61.90%	[97]
<i>MGMT</i>	10q26	DNA repair	22%-39%	[98,99]
<i>TFPI2</i>	7q21.3	Protease inhibitor	46.50%	[100]

methylation associated with HSC activation had been reported at specific loci such as the phosphatase and tensin homologue (*PTEN*) and patched1 (*PTCH1*) genes. These genes were aberrantly methylated in the myofibroblast and associated with the decreased of gene expression<sup>[67,68]</sup>. Our previous study revealed that aberrant promoter methylation of PPAR gamma gene was significantly associated with liver fibrosis in patients with chronic hepatitis B<sup>[69]</sup>. Other genes like Ras GTPase activating-like protein 1 (*RASAL1*) gene were also found to be aberrantly hypermethylated in liver fibrosis<sup>[70]</sup>.

## DNA METHYLATION AND NONALCOHOLIC STEATOHEPATITIS

So far, the relationship between DNA methylation and metabolic diseases was firmly established. Ahrens *et al*<sup>[71]</sup> used array-based DNA methylation and mRNA expression profiling to analyze the liver tissues from patients with non-alcoholic fatty liver disease (NAFLD) ( $n = 45$ ) and health controls ( $n = 18$ ). Aberrant methylation and decreased mRNA expression were seen for nine genes, which included genes for key enzymes in intermediate metabolism (*ACLY*, *PC* and *PLCG1*) and insulin or insulin-like signaling (*IGFBP2*, *IGF1* and *PRKCE*)<sup>[71]</sup>. Studies showed that supplementation of diets lack of methyl donors could induce DNA hypomethylation and the development of steatosis in mice. However, supplementation of diets with methyl donors could prevent the development of NAFLD, suggesting that differences in the DNA methylation status might be a potential factor for individual susceptibilities to hepatic steatosis<sup>[72,73]</sup>. The supplementation of the maternal diet with methyl donors could induce aberrant methylation in adulthood and protect offspring from suffering obesity<sup>[74]</sup>.

## DNA METHYLATION AND LIVER FAILURE

Recent studies found that the aberrant methylation of several genes might participate in the development of liver failure. The aberrant promoter methylation of some anti-inflammatory genes might result in the down-regulate gene expression and inhibit their protective role in liver injury. Our previous study found that glutathione-

S-transferase P1 (*GSTP1*) promoter hypermethylation occurred in patients with acute on chronic hepatitis B liver failure (ACHBLF) which might facilitate oxidative stress associated liver damage<sup>[75]</sup>. A study performed by Fan *et al*<sup>[76]</sup> showed that hypomethylation of IFN- $\gamma$  gene promoter in peripheral blood mononuclear cells might be associated with the onset of ACHBLF. Qi *et al*<sup>[77]</sup> found that the aberrant hypermethylation of glutathione-S-transferase P1 (*GSTM3*) gene occurred in ACHBLF, which was correlated with their disease severity.

## FURTHER PROSPECTS AND SUMMARY

The development of liver diseases is a multifactorial process characterized by the combination and integration of a multitude of alterations including genetic and epigenetic changes. In the past decades, there were exponential increases in the interest and progress of DNA methylation. Studies already revealed the potential role that DNA methylation played in the normal human development and initiation of diseases. DNA methylation-based biomarkers were proposed for disease risk assessment<sup>[78]</sup>, early detection<sup>[79,80]</sup>, prognostic prediction<sup>[81]</sup> and treatment outcome prediction of liver diseases<sup>[82]</sup>. Meanwhile, there was hope for developing therapeutic agents to manipulate aberrant DNA methylation patterns and to treat malignancies<sup>[6]</sup>. In 1970s, Constantinides *et al*<sup>[83]</sup> reported 5-azacytidine had remarkable effects on differentiated states of cells. In 2005, Brueckner *et al*<sup>[84]</sup> reported the drug RG101 could also reactivate tumor suppressor gene by inhibiting human DNA methyltransferase. Therefore, combined genetic and epigenetic information may help clinicians to prevent liver diseases developing in at-risk individuals and from passing on unhealthy DNA methylation characteristics to offsprings.

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