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

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REVIEW

Extended role for insertion sequence elements in the antibiotic resistance of *Bacteroides*

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Abstract

The *Bacteroides* species are important micro-organisms, both in the normal physiology of the intestines and as frequent opportunistic anaerobic pathogens, with a deeply-rooted phylogenetic origin endowing them with some interesting biological features. Their prevalence in anaerobic clinical specimens is around 60%-80%, and they display the most numerous and highest rates of antibiotic resistance among all pathogenic anaerobes. In these antibiotic resistance mechanisms there is a noteworthy role for the insertion sequence (IS) elements, which are usually regarded as representatives of 'selfish' genes; the IS elements of *Bacteroides* are usually capable of up-regulating the antibiotic resistance genes. These include the *cepA* (penicillin and cephalosporin), *cfxA* (cephamycin), *cfiA* (carbapenem), *nim* (metronidazole) and *ermF* (clindamycin) resistance genes. This is achieved by outward-oriented promoter sequences on the ISs. Although some representatives are well characterized, *e.g.*, the resistance gene-IS element pairs in certain resistant strains, open questions remain in this field concerning a better understanding of the molecular biology of the antibiotic resistance mechanisms of *Bacteroides*, which will have clinical implications.

Key words: Antibiotic resistance; Antibiotic resistance genes; *Bacteroides fragilis*; Insertion sequence elements

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SIGNIFICANCE OF *BACTEROIDES*

The species of the genus *Bacteroides* are the most prominent human pathogenic anaerobic bacteria. Additionally, they have other important specialities: they are one of the most important members of the mammalian normal intestinal microbiota and they are the best-studied organisms of a separate and early diverged phylum, Bacteroidetes, of Bacteria. As regards their pathogenic nature, they account for 60%-70% of the total anaerobic pathogens cultivated from clinical samples, and despite the relatively low number of such materials, they often cause high mortality in various infectious processes, such as abscesses and other soft tissue infections, and often cause anaerobic sepsis[1].

Their phylum is a phylogenetic relative of the group of green-sulfur photosynthetic bacteria, the Chlorobiales, and the best-known and most frequently isolated species, *Bacteroides fragilis (B. fragilis)*, as a type species for anaerobic bacteria, is often referred to as the anaerobic *Escherichia coli*[2]. *B. fragilis* was first isolated as '*B. fragilis*' and later renamed as *B. fragilis*. Until the late 1970s, almost all Gram-negative anaerobic bacilli were classified

Table 1 A list of the related species comprising the '*Bacteroides fragilis* group' at present

Bacteroides			*Parabacteroides*
B. acidifaciens	*B. fluxus*	*B. propionifaciens*	*P. distasonis*[1,2]
B. barnesiae	*B. fragilis*[a,b]	*B. pyogenes*	*P. goldsteinii*[1]
B. caccae[1,2]	*B. galacturonicus*	*B. rodentium*	*P. gordonii*
B. cellulosilyticus	*B. gallinarium*	*B. salanitronis*	*P. johnsonii*
B. chinchillae	*B. graminisolvens*	*B. salyersiae*[1]	*P. merdae*[1,2]
B. clarus	*B. helcogenes*	*B. sartorii*	
B. coagulans	*B. heparinolyticus*	*B. stercoris*[1,2]	
B. coprocola	*B. intestinalis*[1]	*B. thetaiotaomicron*[1,2]	
B. coprophilus	*B. massiliensis*[1]	*B. uniformis*[1,2]	
B. coprosuis	*B. nordiia*	*B. vulgatus*[1,2]	
B. dorei	*B. oleiciplenus*	*B. xylanisolvens*	
B. eggerthii[1,2]	*B. ovatus*[1,2]	*B. xylanolyticus*	
B. faecis	*B. paurosaccharolyticus*	*B. zoogleoformans*	
B. finegoldii	*B. plebeius*		

[1]The main pathogenic species of *Bacteroides* that were included in an antibiotic susceptibility study and are most frequently isolated from clinical specimens; [2]The 10 *Bacteroides* species earlier comprising the *B. fragilis* group are marked with a superscript b.

in this genus, and only the more recent molecular techniques, such as DNA-DNA homology measurements and 16S rRNA sequence comparisons, allowed a more accurate classification. Thus, the genera of *Bacteroides*, *Prevotella* and *Porphyromonas* were formed from the earlier *Bacteroides* genus during the late 1980s. 16S rRNA phylogeny and other molecular classification methods were then applied, making the picture more diverse[3]. The parent genus *Bacteroides* now contains 41 described and well-characterized species. Some other former *Bacteroides* species were reclassified into the newly formed *Parabacteroides* genus[3], which belongs in the *Porphyromonadaceae* family (*P. distasonis* and *P. merdae*) (Table 1). *Bacteroidaceae*, *Marinilabiaceae*, *Porphyromonadaceae*, *Prevotellaceae* and *Rikenellacaea* are the families of the Bacteroidales order. Together with other important but aerobic taxa (the Cytophagales, Flavobacteriales and Sphingobacteriales orders), they form the Bacteroidetes phylum. The current situation regarding the species of the *Bacteroides* and *Parabacteroides* genera, the subjects of the current review, is summarized in Table 1 with some implications in respect of their pathogenic potential. In a recent study, the phylogenetic relations between *Bacteroides* species were analysed by multilocus sequence analysis, and thus these species could be ranked into 10 subgroups also showing some common characteristics regarding their pathogenic nature and sites of isolation[4].

Genomic studies have revealed the important genetic characteristics of this group of anaerobic bacteria and contributed to extensive metagenomic analyses of their habitat, the intestines and the participating microbiota[5]. These studies have reconfirmed that *Bacteroides* species are important symbionts there and opened up new ways for the investigation of this firmly interacting ecosystem. Besides the earlier cultivation and microscopic methods, metagenomic analyses have also proved that the two most abundant taxa there are Bacteroidales and Firmicutes (low G + C Gram-positives)[5,6]. The composition of the mammalian intestinal microbiota depends on the type of food intake (herbivorous, carnivorous or omnivorous)[7], but in the case of human beings three enterotypes can be distinguished as regards the prevalence of the main abundant constituents (*Bacteroides*, Prevotella and *Ruminococcus*); it is suspected that this is determined by the host and does not depend on the geographic origin[8]. The *Bacteroides* as one of the groups of predominant constituents of the human intestines exert beneficial effects for the host. However, experiments involving the application of metagenomics suggest that the intestinal microbiota, including *Bacteroides*, can affect not only the food intake, but also the development and physiology of the intestines and the immune system, and such distant organs as the liver, muscles, circulation and central nervous system. Thus, their roles regarding participation in illness states such as obesity and inflammatory bowel diseases have been the subjects of previous and ongoing investigations[9-11].

Virulence mechanisms of Bacteroides spp.

Though *Bacteroides* can be regarded as only opportunistic pathogens since they reside in the intestines in high cell numbers and cause diseases with underlying predisposition circumstances such as trauma, circulation defects and immunosuppression, they usually possess a pathogenic repertoire with which they participate in infections. *B. fragilis*, the earliest identified and thus the type species, is isolated most frequently from anaerobic infections with a prevalence of 60%-70%. As it is estimated to have a prevalence in the intestines of only 0.5%-5.0% and to be localized to the epithelium rather than to the lumen, it can be regarded as the most pathogenic species among the *Bacteroides*, and this is supported by the experimental data[1]. The most frequent infections that it causes are intra-abdominal and intra-pelvic, lung and brain abscesses, appendicitis, diarrhea, inflammatory bowel disease, lower respiratory and soft-tissue infections, and sepsis. The main predisposing factors are usually surgery, mixed aerobe-anaerobe infections, immunosuppression, diabetes and circulatory defects. However, besides the prominent pathogenic role of *B. fragilis,* most *Bacteroides* are capable of adhering, evading and destroying the tissues with their direct and indirect virulence mechanisms, which are production of capsules, fimbriae and adhesins, tissue destruction enzymes (fibrinogenases, haemolysins, neuraminidase and enterotoxin) and properties of aerotolerance, evasion of the host immune system, and antibiotic resistance mechanisms[1].

The most potent virulence mechanism of *B. fragilis* has been demonstrated to involve certain capsular polysaccharide (CPS) species[12]. In the mid-1980s, the use of CPS material of *B. fragilis* was shown to evoke abdominal abscesses experimentally in a rat model, and the nature of the immuno-modulation involved the induction of a humoral response[13]. The chemical structures

of two CPS species, CPS-A and B, of *B. fragilis* NCTC 9343 were later determined and their abscess-inducing properties were proved to be due to a zwitterionic structure[14,15]. The capsules participate in immuno-modulation by other usual modes of interactions, the inhibition of phagocytosis and complement action. Electron microscopically, *B. fragilis* may be seen to have small or large capsules or only an electron-dense layer which is implicated in complement resistance[16-18]. Studies have led to the cloning of these CPS operons[19,20], and subsequently altogether 8 operons with similar structures have been found in the genomic sequence of *B. fragilis* NCTC9343 that have a common regulatory property, the possession of invertible promoters[21]. This special regulatory feature may result in numerous variable surface compositions through activation of on-off switches (about 2^8) in the case of a single strain. The examination of *B. fragilis* YCH46 and 638R genomes demonstrated that at least 10 CPS operons can be located on these genomes, which may have different alleles (n = 28), allowing a much higher number of variations in possible surface compositions[22]. The large and small capsule phenotypes are suspected of being regulated by the expression of the gene *BF2782* (or *BF2790* in *B. fragilis* 638R), which is a putative sugar transferase participating in the synthesis of the CPS species and is also the subject of invertible promoter structure[22]. Similar CPS operons are suspected of functioning under the regulation of invertible promoters in other *Bacteroides* species (*B. caccae*, *B. ovatus*, *B. thetaiotaomicron*, *B. uniformis*, *B. vulgatus*, *P. merdae* and *P. distasonis*), but not in the oral *Bacteroides* relatives (*Prevotella* and *Porphyromonas*)[23]. The CPS-A of *B. fragilis* is capable of regulating the maturation of the immune system which, in turn, is an important contribution to the overall symbiotic interactions between *Bacteroides* and the host[24,25].

Another important virulence factor is the enterotoxin of *B. fragilis*, which may cause diarrhea especially in young mammalians. This enterotoxin is a metallo-protease capable of the specific cleavage of the E-cadherin protein in the zonula adhaerens portion of the intestinal epithelium. This causes specific processes leading to the symptoms of diarrhea; disorganization of the actin cytoskeleton, epithelial fluid loss, inflammation, and possible penetration of the enterotoxinogenic *B. fragilis* cells into the nearby and distant tissues[26]. The inflammatory action of the *B. fragilis* enterotoxin may be so pronounced that the malignant transformation it causes can be detected both clinically and experimentally[27,28]. The genes of the enterotoxin, consisting of three main types (*bft1-3*), lie on a specific portion of a 'pathogenicity island', which is a conjugative transposon resembling other *B. fragilis* genome-borne conjugative transposons[29-31]. The similar CTn*86* and CTn*9343* elements have molecular variants resulting from (1) insertion of a ca. 6 kb region containing the *bft* genes into CTn*86* (accordingly, *bft*-positive CTn*86*s are enterotoxinogenic, whereas *bft*-negative CTn*86*s are not); (2) replacement of the 3' regions of both CTns; and (3) insertion of a novel ca. 7 kb region into some CTn*9343*s[32,33]. A more detailed summary of the pathogenicity and virulence factors of *Bacteroides* is to be found in an excellent recent review[1].

Antimicrobial resistance of Bacteroides spp. and its genetic background

As the *Bacteroides* are the most significant human anaerobic pathogens, detection of their antimicrobial susceptibilities has a significant history, and trends have been observed in the most frequent resistance rates and the most numerous resistance mechanisms among their clinical isolates. As time has passed, these latter resistance trends have become more pronounced. In the 1960s and 1970s, the strains were much less resistant to all groups of antibiotics than more recently[34]. In the meantime, the recommended susceptibility measurement methods have changed. Since the 1980s, the recommended method for the detection of their antibiotic susceptibilities has been agar dilution[35]. Regular studies have been carried out, especially in the United States and in Europe[36,37], and the breakpoint recommendations of the NCCLS (National Committee for Clinical Laboratory Standards, currently the Clinical Laboratory Standards Institute-CLSI, www.clsi.org) in the United States have been widely used for resistance categorization; additionally, we now have the recommendations of another influential body, EUCAST (www.eucast.org). Since the *Bacteroides* in the intestines are readily exposed to antibiotics administered orally and excreted into the bile, a continuous increase in resistance rates has been observed for all major antibiotics. The resistance to tetracycline has changed most profoundly, which may be explained by the intensive use of tetracycline and the fact that the spread of tetracycline resistance elements is highly enhanced by tetracycline (see the explanation below)[34].

The *Bacteroides* have displayed a significant rate of resistance to 'normal β-lactams' (penicillins and 1st and 2nd generation cephalosporins) throughout the studied periods, but some increases have also been observed. One important issue relating to the 'normal β-lactam' resistance is the breakpoint categorization, since the MIC values for all such drugs are scattered widely, ranging from the low 0.25 μg/mL to the very high 256 μg/mL. Thus in a 1990 European study, only 12% of *B. fragilis* strains were found to be resistant to ampicillin at a breakpoint of 32 μg/mL[38], whereas in a study in 2000 with 2/64 μg/mL as breakpoints, 99.3/27% were categorized as resistant[39]. It was additionally observed that the distribution of 'normal β-lactam', especially ampicillin, resistance distribution is bimodal, with modes at about 32 and ⩾ 256 μg/mL[38]. Since the majority of *Bacteroides* isolates exhibit β-lactamase activities, this was proposed to be the main resistance mechanism[34]. The gene *cepA* of an Ambler Class A β-lactamase is very prevalent[40] (about 70%, according to our own unpublished observations) among *B. fragilis* and other *Bacteroides* strains. Little is known concerning the mechanisms of

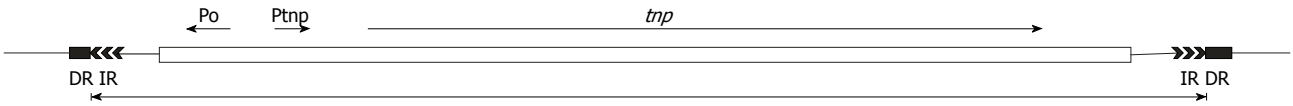

Figure 1 Schematic structure of an insertion sequence element. DR: Direct repeats; IR: Inverted repeats; Po: Outward-oriented promoter; Ptnp: Promoter of the transposase. The transposase gene is denoted as tnp. The borders of the insertion sequence (IS) are indicated by the closed arrowheads below.

resistance to β-lactam/β-lactamase inhibitor combinations; however, the rates in the United States (< 1%) and in Europe (10.3%) have been increasing continuously in recent years, probably because of the extensive use of such drugs[36,37].

Cefoxitin was earlier a very effective antibiotic for the treatment of *Bacteroides* infections, but the levels of resistance to this drug rose by 6% and 10.3% in Europe and the United States, respectively[39,41], during the 1990s, though this has been followed by a decrease (12% *vs* 9%) in the past decade in the United States[36]. The main resistance mechanisms involve the decreased affinity of the penicillin-binding proteins to cefoxitin and the production of another Ambler Class A β-lactamase capable of hydrolyzing cephamycins[42]. The gene for this latter β-lactamase, *cfxA*, has been located on a mobilizable transposon MTn*4555*[43], which has been proved to have several variants at its 3' end[44,45].

Some *B. fragilis* isolates are also resistant to the wide-spectrum carbapenems, due to a metallo-β-lactamase coded by the *cfiA* (*ccrA*) gene[46,47]. Despite the low prevalence of carbapenem-resistant *B. fragilis* isolates (about 1%), this has displayed a continuous rise since the introduction of these drugs[37,41]. It is very interesting that the *cepA* and *cfiA* genes are found mutually exclusively among *B. fragilis* isolates and define two genetic groups (Division I -cepA-positive and Division II -*cfiA*-positive)[48,49] that can also be differentiated by the levels of DNA-DNA homologies[50], PCR typing methods[48], ribotyping[51], multi-locus enzyme electrophoresis[49] and MALDI-TOF mass spectrometry[52,53].

The rate of resistance to the macrolide-lincosamide-streptogramin antibiotics of 32.4% is not an exception; the rates of resistance presumed to be caused by *ermF* genes has a steep rise[37]. Our recent investigations based on susceptibility measurements and resistance gene detection (unpublished) of clinical *Bacteroides* isolates revealed that other resistance genes (*ermB*, *ermG*, *mefA* and *msrSA*)[54,55] may participate significantly in the development of clindamycin resistance.

Resistance to 5-nitroimidazoles is caused either by alterations in the cellular redox system that can diminish the lethal action of these drugs or by 5-nitroimidazole reductases that reduce the nitro group of 5-nitroimidazoles to an amino group without the formation of toxic intermediates[56-61]. The 5-nitrimidazole reductases are coded by *nim* genes that bear about 60%-70% mutual homologies and have 9 representatives (*nimA-I*)[60,62-64]. The form *nimI* has been found only among *Prevotella baroniae* isolates, while some *Bacteroides*-specific *nim* genes are present in other source organisms too[65-69]. The rates of resistance to metronidazole are fortunately very low among *Bacteroides* strains in most places (< 1%).

Tetracycline resistance has been estimated to be approaching 100%; the resistance gene is *tetQ*, coding a ribosomal protection protein. The *tetQ* genes are found on conjugative transposons[70]. Interestingly, *Bacteroides* carry another tetracycline resistance gene *tetX* (or its amino-terminally truncated, 60% homologous variant *tetX1*)[71], which is capable of oxidizing the tetracycline molecule[72], but since it requires oxygen for this process, its role in the tetracycline resistance of *Bacteroides* is very limited. *Bacteroides* is resistant at a low level (1.7%) to tigecycline, a synthetic minocycline, glycylcycline derivative. In such tigecycline-resistant cases, a direct role of the *tetX* and *tetX1* genes has not been confirmed for the *Bacteroides*[73].

The *Bacteroides* are now becoming resistant to the once fully effective fluoroquinolones such as trovafloxacin and moxifloxacin, reaching resistance rates of > 40% and 13.6% in the United States and Europe, respectively. Additional data on the antibiotic resistance rates of *Bacteroides* are to be found in a recent exhaustive review[74].

INSERTION SEQUENCE ELEMENTS

There are a huge variety of transposable and conjugally mobile genetic elements, in particular among prokaryotes. IS elements are short (from 600 to 2000 bp long), double-stranded integrative DNA sequences that code for only a transposase gene, bordered by inverted repeat sequences; during their integration, they usually cause target site duplications of a small number of nucleotides. A general scheme relating to their organization is presented in Figure 1. They are to be found in all three domains of life (Archaea, Bacteria and Eukarya). Their classification is based on the ends of their inverted repeats and the conserved amino acid residues of the transposase genes[75]. In this way, about 25 families are distinguished among prokaryotes and are usually named after their earliest and best-examined members. Some families fulfil the above-mentioned description criteria, but molecularly represent a more divergent type of elements, *e.g.*, the application of different transposition mechanisms to the main groups of IS elements which harbor transposases with an indispensable aspartate-aspartate-glutamate (DDE) motif forming the active catalytic center. Similar motifs can be found in the integrase proteins of retroviruses and, among others, in

Table 2 The 5-nitroimidazole resistance *nim* genes of interest for *Bacteroides*

Nim gene type	Carrying genetic element	Activating IS	No. of isolates[1]
nimA			
	pIP417 (7.7 kb)	IS*1168*	10[102,114,115]
	10 kb uncharacterized plasmid	IS*1168*	2[102]
	8.2 kb uncharacterized plasmid	IS*614*	1[102]
	Chromosomal	IS*1168* or Unknown	3[102]
	Unknown	IS*1168*	12[115]
	Unknown	IS*1169*	1[116]
nimB			
	Chromosomal	IS*1168* or IS*612* or IS*614*	8[102,114]
	Unknown	IS*1168*	3[116]
nimC			
	pIP419 (10 kb)	IS*1170*	4[115]
	Chromosomal	IS*1170*	2[102]
	Unknown	IS*1170*	2[63,116]
nimD			
	pIP421 (7.3 kb)	IS*1169*	1[102,117]
	Chromosomal	Unknown	1[102]
	Unknown	IS*1169*	6[116]
nimE			
	pBF388c (pWAL610, 8.3 kb)	IS*Bf6*	5[102,118]
nimF			
	Chromosomal	Unknown	1[116]
nimG			
	Unknown	Unknown	1[63]
nimH			
	Unknown	Unknown	-[2]

[1]The number if isolates with the given genotypes are indicated with references; [2]Taken from GenBank (www.ncbi.nl.nih.gov, acc. no. FJ969397).

RNase H ribonuclease, in the DNA polymerase I 3'-5' proofreading activity domain and in the RuvC recombinase proteins of bacteria forming the RNase H enzyme superfamily. The reader can find further data on the classification and transposition mechanisms in some older and more recent reviews[75,76] and in the IS Finder database (www-is.biotoul.fr)[77].

The simple genetic organization in *sensu stricto* is sufficient for IS element dispersal, and they can therefore be regarded as appropriate examples of selfish genetic elements. However, thorough examinations of their prevalence, genetic structure and transposition suggest that they not only parasitize their hosts, but sometimes participate in firm interaction with them. Such interaction with the host may be accomplished *via* (1) a promoter supply for the host genes; (2) increased evolution rates; and (3) a metabolic load. The activation of the expression of nearby genes by promoter supply is mediated by outward-oriented promoters and is specific for a small subset of ISs. This way, various bacterial genes can be activated, resulting in antibiotic resistance most notably, and the ISs act among others on various antibiotic resistance genes, *e.g.*, *bla*$_{TEM(pBR322)}$ of *Escherichia coli*[78], *bla*$_{OXA-51}$ *Acinetobacter baumannii*[79] and *oprD* of *Pseudomonas aeruginosa*[80]. They can influence the evolution potential of their host by their mutagenization of the host genomes by hopping activity[81]. It is also known that the introduction of a copy of an additional accessory genetic element, *e.g.*, a plasmid, and the amplification of their physiological copy number means a fitness decrease first and then an adaptation[82], which is also valid for IS elements. IS elements participate in activation of *Bacteroides* antibiotic resistance genes.

The discovery that erythromycin and clindamycin resistance is due to an MLS$_B$ resistance mechanism (capable of causing cross-resistance to the chemically different macrolid, lincosamide and streptogramin B antibiotics), mediated by *ermF* genes, and the subsequent linking of these genes to similar compound transposons, was the first indication of IS element involvement in antimicrobial resistance among *Bacteroides*. Clindamycin resistance plasmids were first detected in clindamycin-resistant isolates[83]. Such plasmids as pBF4 (pIP410), pBFTM10 (pCP1) and pBI136 were characterized very well molecularly in the 1980s[84]. pBF4 (41 kb) harbored Tn*4351* bordered by inverted copies of IS*4351* and in between *ermF* and an aerobic-type tetracycline resistance gene, *tetX*[85]. Tn*4551* accounts for a large portion (about 30%) of pBFTM10 (15 kb) and contains *ermF* in direct repeats of IS*4351*[84]. pIB136 (80 kb) also contains Tn*4551*, but with a high preponderance to lose this structure[86].

The 1990s revealed other important links between IS elements and the antibiotic resistance of *Bacteroides*. After cloning of the determinant for the carbapenem resistance, *cfiA*[46,47], PCR detection and parallel molecular methods have demonstrated that carbapenem-resistant mutants can arise in single-step mutations from *cfiA*-positive but carbapenem-susceptible *B. fragilis* isolates[87], which proved to be insertions of IS*1186*[88] and IS*942*[47]. Later studies confirmed these findings and the roles of a series of other IS elements were identified in carbapenem-resistant strains from such different geographic regions and countries as Europe (France, the United Kingdom, Hungary, Sweden, Switzerland, Norway and Italy), the United States, Japan, Korea and Kuwait[48,89-95]. The 5-nitroimidazole resistance genes, *nim*s, also carry various IS elements in their upstream regions. In these cases, the *nim* gene types, the carrying genetic elements and the activating ISs were linked (Table 2). The presence of IS elements has been demonstrated in the upstream regions of other β-lactamase genes, *cepA* and *cfxA*. In a high cephalosporinase-producer strain, *B. fragilis* CS30, this feature was caused by a specific DNA sequence that contained an IS21-like region (IS*Bf1*) at its 3' end adjacent to the *cepA* gene[96]. In the case of a representative strain (*B. vulgatus* CLA341) for *cfxA*-mediated cephamycin resistance, the upstream region of the *cfxA* gene also contained an IS element (IS*Bf8*) that was identified later by bioinformatics analysis[97,98]. The majority of the *cfxA* genes of *Bacteroides* do not normally contain this (IS*Bf8*) and another mobile element (MITE*Bf2*) in their upstream region[45]. Interestingly, again in high β-lactamase-

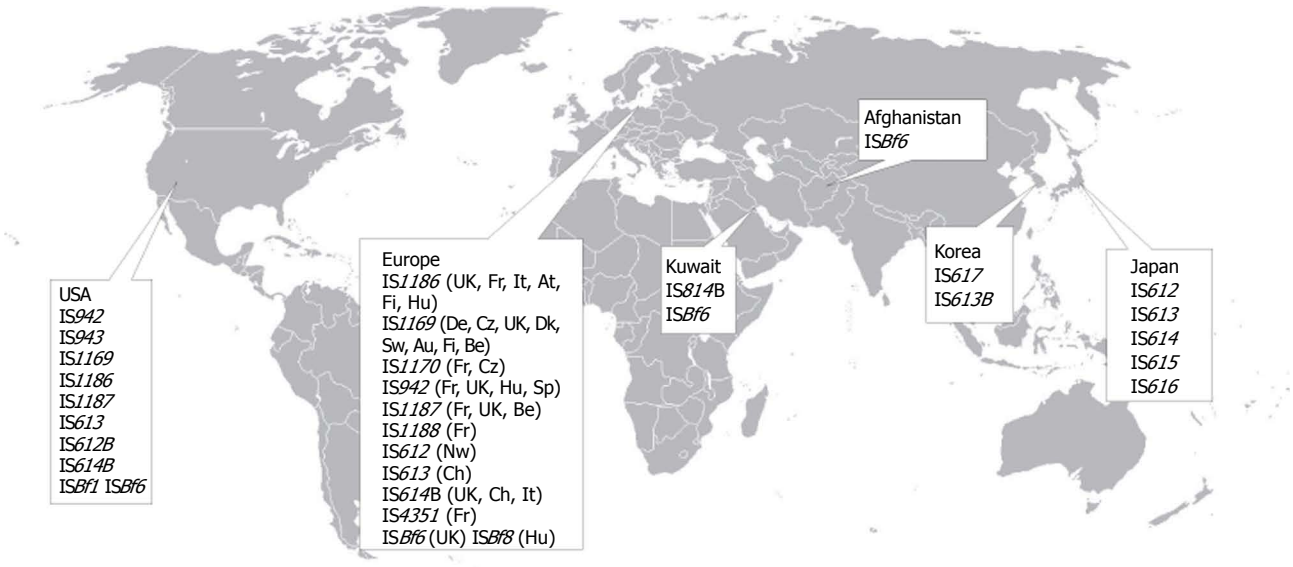

Figure 2 Insertion sequence elements found worldwide in antibiotic-resistant *Bacteroides* isolates. For Europe, the following abbreviations are used to identify the countries in which the insertion sequences were isolated: At: Austria; Be: Belgium; Ch: Switzerland; Cz: Czech Republic; De: Germany; Dk: Denmark; Fi: Finland; Fr: France; Hu: Hungary; It: Italy; Nw: Norway; Sp: Spain; Sw: Sweden; UK: United Kingdom.

producer *cfxA*-positive strains, the presence of IS*614*-like elements has been revealed in the upstream region of the resistance gene by inverse PCR[44].

For *cfiA* and *cfxA,* a heterogeneous resistance phenotype has been detected by diffusion methods (especially the Etest) in strains that have elevated agar dilution MICs and do not have IS elements in the upstream region of the resistance genes[45,99].

Though specific for some representative strains and resistance genes, the *Bacteroides* IS elements have been shown to be capable of activating all IS-requiring resistance genes. In this way, IS*4351* can activate *cfiA*[48], the IS elements of the *nim* and *cfiA* genes are interchangeable, and an IS element discovered for *cfiA*, IS*614* (or its variant), has been found to activate the *cfxA* gene also[44]. However, little is known about the prevalence and epidemiology of the resistance gene-activating ISs apart from their being found in resistant isolates. The best-studied examples are the *nim* and *cfiA*-carrying strains, but these differ considerably with respect to the prevalence of 'silent' and activated genes. Thus, all well-characterized *nim* genes are associated with an IS element (Table 2), but the majority of the *cfiA* genes are 'silent', and not associated with ISs[52,87,89,100,101]. Examinations of the insertion sites of ISs among *nim* and *cfiA* genes revealed that for *nim*s the insertion sites are well defined and conservative for a particular *nim* gene type[102], whereas for the *cfiA* genes they vary[91,92]. This means a well-known mechanism for the emergence of nitroimidazole resistance by the *nim* and IS combination, which was investigated and discussed recently, especially for the β-lactam resistance mechanisms of *Enterobacteriaceae*[103,104], involves the consecutive steps of emergence, adaptation and spreading. For the *Bacteroides* the *nim* gene IS combinations first emerged, which were then inserted into specific replicons (plasmids and chromosomes) and subsequently were spread in the *Bacteroides* population.

Another epidemiological concern besides the interchangeability of the IS elements is their geographical distribution (Figure 2). A number of studies of IS elements in resistant strains indicated that there is little geographical restriction to their spreading worldwide, *e.g.*, IS*614* or IS*614*-like elements were found ubiquitously, though some local tendencies can also be observed (Figure 2, cf. Japan and Korea). It could also be that these IS elements vary in the nucleotide sequence, giving rise to isoforms (not mentioned in full detail here) and could be mosaics/combinations of other elements. This can be explained by the homologous nature of these elements and the fact that they can be harbored coincidentally in an unknown proportion of the strains.

While the role of IS elements in emerging antibiotic-resistant *Bacteroides* strains is well documented, the process of the movement/skipping of the IS elements from their proper positions has been investigated only poorly. Podglajen *et al*[87] studied this process *in vitro* and reported a rough estimation of the development of imipenem-resistant strains, with 10^{-8} to 10^{-7}/cell frequencies in a given culture. Edwards *et al*[89] detected this process *in vivo* when the initially susceptible strain in a patient with a *B. fragilis* infection treated with imipenem became resistant[105].

Overall, the IS elements found among *Bacteroides* species belong in 9 IS families, members of 5 families being capable of activating antibiotic resistance genes. An overview of these elements is provided in Table 3.

THE PROMOTERS CARRIED BY THE BACTEROIDES IS ELEMENTS

Although IS element insertion correlates well with an-

A Consensus sequences

Regions	-33	-7
	TtTG	tnnTAnnTTTGY

B

Gene	IS element			
cepA	IS*Bf1*	TTTG	16 nt	TcaTAccTTTGTtgga˅
cepA	-	aTTGaaTT	16 nt	TcaTAccTTTGTtgga
cfxA	IS*Bf8*	TTTG	17 nt	atgTAccTTTGTcggc˅
cfxA	-	TTTc	10 nt	atgTAccTTTGTcggc
cfiA	IS*942*	agTG	17 nt	TtgTActTTTGCca˅
	IS*1186*	TTTG	16 nt	gctTAacTTTaCgcaa˅
	IS*1187*	TTG	17 nt	gacgAatTTTGCa˅
	IS*1188*	TTG	17 nt	TtgTAtcTTTGCaca˅
cfiA	-	TaTa	8 nt	atgTtagTTTGaatac˅
ermF	IS*4351*	aTTG	18 nt	TtaTAtgTTTGCtca˅
nimA	IS*1168*	TTTG	18 nt	gctTAacTTTaCgca˅
tetQ		TTTG	16 nt	gtgTAatTTTGTaatc˅

Figure 3 The nucleotide sequences of the promoters of some important Bacteroides antibiotic resistance genes. The consensus sequence with the conserved regions (A), and the actual promoter sequences (B). The match with the consensus is shown in bold capital letters, proven transcriptional initiation sites are marked in bold with an arrowhead next to them; n denotes any nucleotide, and small letters in the consensus indicate less conserved bases. The own promoters of *cepA* and *cfxA* were searched for bioinformatically and are not IS elements next to them in the list. The own promoter sequence of *cfiA* is from our unpublished preliminary experiments made by 'rapid amplification of cDNA ends' capable of amplifying in PCR the 5' end of the mRNA. Underlined -33 regions are parts of compound promoters and these parts originate only from insertion sequence elements in the cases of these promoters.

tibiotic resistance gene expression, the main reason for their up-regulation is that the IS elements carry outward-oriented promoters capable of driving the expression of the genes. The initial hypothesis for this up-regulation was the lending of IS activation mechanisms from other antibiotic resistance genes for aerobic species, but only *E. coli* promoter sequences could be investigated for these *Bacteroides* IS elements at that time. There was a straightforward result concerning the requirements for transcription in *Bacteroides* when Bayley *et al*[106] recognized the nucleotide composition of the promoter sequences for several antibiotic resistance and other *Bacteroides* genes. The promoter consensus sequence for *Bacteroides* is depicted in Figure 3A. This highly different requirement in promoter sequence was later explained by the results of Vingadassalom *et al*[107], who proved that the primary σ subunit of the *Bacteroides fragilis* RNA polymerase is unusual and different from that of other bacteria; whereas it is able to start transcription from original *Bacteroides* promoters in reconstitution experiments, it clusters firmly together with the suspected primary σ-factors of other Bacteroidetes species, but only distantly to the primary and stationary σ-factors of other bacteria.

Several antibiotic resistance gene promoter sequences were recognized during these investigations. The first was for the *cepA* gene of *B. fragilis* CS30 in the original work of Bayley *et al*[106] Similarly, those authors determined the promoter for the *cfxA* gene on MTn*4555* of *B. vulgatus* CLA341[96]. Interestingly, this promoter is a compound one: the -7 region originates from a prototype MTn*4555* backbone and the -33 region from an IS element (IS*Bf8*). MTn*4555* insertion of the IS*614* elements is associated with increased resistance to cefoxitin, though the exact transcription initiation site for this IS element and the promoter remain to be elucidated[44]. Among these rare data relating to the promoter structures of *Bacteroides*, those carried by IS*612*, IS*613*, IS*614*, IS*615* and IS*616* elements activating the *cfiA* genes have been recognized, thereby furnishing us with important confirmatory data[90,93]. Podglajen *et al*[108] also determined the outward-oriented promoters of some important IS elements (IS*1186*, IS*942*, IS*1187* and IS*1188*) participating in activation of the *cfiA* genes of some carbapenem-resistant *B. fragilis* isolates. Although the recognition of the requirements for the *Bacteroides* promoter nucleotide sequence facilitated an understanding of their antibiotic resistance mechanisms, there was also research into other aspects of their properties, *e.g.*, the CPS on-off regulation[21]. Figure 3B lists the known and some proposed sequences of promoters of antibiotic resistance genes of *Bacteroides*.

Table 3 The insertion sequence elements involved in the up-regulation of antibiotic resistance genes in *Bacteroides*

IS family[1]	Group[1]	IS[2]	Activated genes
IS4	ISPepr1		
		IS*943*	*cfiA*
		IS*Bf8*	*cfxA*
IS5	IS5		
		IS*1186* (IS*1168*)	*cfxA, cfiA, nimA, nimB*
		IS*1169*	*cfiA, nimA, nimD*
	IS1031		
		IS*Bf6*	*nimE*
IS21	-		
		IS*Bf1*	*cepA*
IS982	-		
		IS*1187*	*cfiA*
IS31380	IS942		
		IS*942*	*cfiA*
		IS*1170*	*nimC*
		IS*612*	*cfiA, nimB*
		IS*613*	*cfiA*
		IS*614*	*cfxA, cfiA, nimB*
		IS*615*	*cfiA*
	-		
		IS*1188*	*cfiA*
		IS*4351*	*ermF, cfiA*
		IS*616*	*cfiA*

[1]The IS families and the subgroups within them (taken from IS Finder)[77]; - indicates no further classification; [2]The species of IS elements activating the resistance genes of *Bacteroides* spp.; the mosaics and isoforms are not indicated. IS: Insertion sequence.

Other resistance mechanisms

Despite the dominance of IS element-borne activation of the antibiotic resistance genes of *Bacteroides*, natural resistance (to aminoglycosides, 1st and 2nd-generation fluoroquinolones and aztreonam), resistance emerging by point mutations, and the enforcement of internal regulatory mechanisms of the genes should be mentioned.

Point mutations in the *gyrA* gene (coding for a subunit of topoisomerase Ⅱ) can cause ciprofloxacin, moxifloxacin and trovafloxacin resistance[109]. A special, well-characterized resistance mechanism of *Bacteroides* is coded by tetracycline resistance conjugative transposons harboring the *tetQ* genes. The *tetQ* genes have their own promoters that can be up-regulated by tetracycline, as observed in the 1970s and exhaustively analyzed since the 1990s[70]. This is mediated by an attenuation mechanism where the transcription stalls at a leader upstream of *tetQ* in the absence of tetracycline, but in the presence of tetracycline the transcription proceeds. The *tetQ* gene is in an operon with the regulatory proteins of *rteABC*, which upon tetracycline exposure up-regulate the excision, mobilization and conjugation genes[110]. For this regulation to be effective, other regulatory processes are also involved, whose absence makes the conjugative transposons constitutive with respect to tetracycline[111]. Some other important resistance genes code efflux pumps, *e.g.*, *bexA* (fluoroquinolones)[112], *mefA* and *msrSA* (clindamycin)[55], and an endogeneous efflux mechanism, mediated by the *bmeABC* genes, can be up-regulated by mutations in the amino acid sequence of the coded effector proteins[113].

CONCLUDING REMARKS

Bacteroides species are noteworthy participants and contributors to human health and disease. They comprise a group of bacteria with additional molecular biological specific features as regards their promoter and RNA polymerase structures and a huge number of surface variations due to the invertible promoters at their CPS operons. The regulation of their antibiotic resistance genes is in most cases also specific; they need up-regulatory IS elements for antibiotic resistances to develop. However, there is a paucity of data about the observed associations in resistant strains: the promoters of less characterized IS elements are still to be determined, the roles of up-regulatory IS elements in other resistance genes could be investigated, and the frequencies with which the IS elements move to the upstream positions of the resistance genes could be examined in greater detail. These latter approaches would promote a better understanding of the whole picture of the rather prevalent antibiotic resistances of the *Bacteroides* species, which in turn would facilitate the design of better antimicrobial therapies against this important group of bacteria in the future.

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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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Patent (list all authors)

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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Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc*. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc*.

Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc*.

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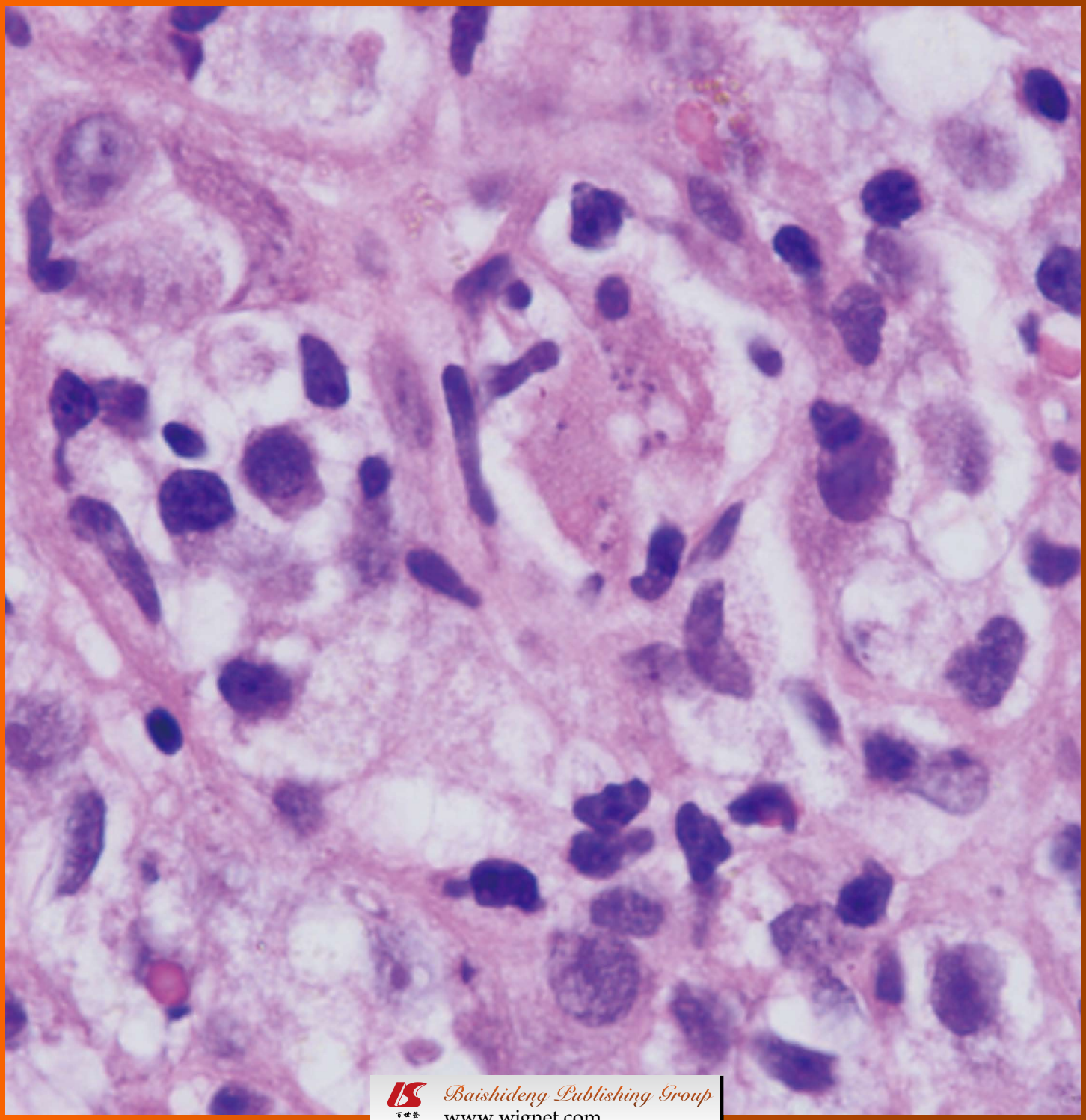

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BRIEF ARTICLE

Natural contamination of human hands with enteric parasites in Indian Subcontinent

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Abstract

AIM: To investigate the prevalence of enteric parasite contamination on hands and the potential role naturally contaminated hands may have in their transmission.

METHODS: Prior to initiating the survey, the protocol was reviewed and approved by respective Institutional Review Boards of each survey site (Dhaka, Bangladesh and Kolkata, India). Both stool and corresponding hand wash samples collected, were analyzed for the presence of enteric parasitic ova/(oo)cysts employing conventional microscopy coupled with permanent staining techniques. Additionally molecular approaches such as polymerase chain reaction (PCR) of enteric parasites recovered from both stool and corresponding hand wash samples, were also used to further confirm their identity.

RESULTS: A total of 972 stool samples were collected from both sites surveyed (300 volunteers from Kolkata, India and 672 from Dhaka, Bangladesh). Parasitic analysis revealed, 113 (38%) from Kolkata, India and 267 (40%) of stool samples from Dhaka, Bangladesh were positive for parasitic ova/(oo)cysts. When the corresponding hand wash samples were analyzed, 43 (14%) stool-positive volunteers in Kolkata, India and 47 (7%) in Dhaka, Bangladesh were positive for enteric parasitic ova/(oo)cysts. *Ascaris lumbricoides* (*A. lumbricoides*) ova and *Giardia lamblia* (*G. lamblia*) cysts predominated in hands wash samples from both sites surveyed (from India, *A. lumbricoides* ova, 53%; *G. lamblia* cysts 31% and from Bangladesh, *A. lumbricoides* ova, 47%; *G. lamblia* cysts 19%). Genotypic analysis of enteric parasitic ova/(oo)cysts obtained from both stool and corresponding hand wash samples taken from the same person were found to be identical.

CONCLUSION: These results suggest a possible role of hands contaminated with enteric parasites' ova/(oo)cysts in the transmission of these parasites highlighting another role of hand hygiene/proper hand washing in reducing the disease burden in low socio-economic communities.

Key words: Enteric parasites; *Ascaris lumbricoides*; *Giardia lamblia*; Natural contamination of hands

Core tip: The authors report contamination of human hands with enteric parasites in two independent sites surveyed in two developing countries of the Indian

Subcontinent. This study indicates that contamination of hands with parasite ova/(oo)cysts are common among those populations already infected and this may play a role in continued cycle of transmission and/or re-infection within the community.

Ijaz MK, Talukder KA, Aslam M, Haque R, Ganguly S, Azmi IJ, Hossain MS, Mukherjee AK, Raj D, Ahmed I, Kamal J, Rubino JR, Nur-E-Kamal A. Natural contamination of human hands with enteric parasites in Indian Subcontinent. *World J Clin Infect Dis* 2013; 3(2): 13-19 Available from: URL: http://www.wjgnet.com/2220-3176/full/v3/i2/13.htm DOI: http://dx.doi.org/10.5495/wjcid.v3.i2.13

INTRODUCTION

Parasitic infections are a global problem. Worldwide, more than a billion people are estimated to be infected with just one species of parasite [(*Ascaris lumbricoides* (*A. lumbricoides*)], mostly in derdeveloping countries[1,2]. Human association with enteric parasites extends into human history[3-5]. Some of these enteric parasitic agents, also called neglected intestinal parasites are responsible for causing not only chronic infection predisposing to malnutrition in children thereby lowering their resistance to infectious diseases, but also lead to malabsorption and further malnutrition by impairing intestinal absorption of nutrients critically required for child's growth and cognitive development[6,7]. This leads to the development of a vicious cycle of malnutrition - enteric pathogens - malnutrition synergy. For example, *A. lumbricoides*, a soil transmitted helminth (STH) sheds up to 200000 ova per day in the feces of infected person. With ineffective collection and treatment of human waste particularly in developing countries, *A. lumbricoides* ova widely contaminate the environment essentially maintaining a vicious cycle of malnutrition - enteric parasite - malnutrition synergy in human populations living under un-hygienic conditions as seen in most developing countries today[8,9]. Parasitic infections in humans are usually transmitted through fecal-oral route, using vehicles such as food, water environment, and hands contaminated with protozoa (oo)cysts and nematode ova, includes *Cryptosporidia*, *Giardia*, *Entamoeba histolytica*, *Enterobius* (pinworms), and *Ascaris* (roundworms)[10,11]. Such infections lead to loss of appetite, impaired digestion, malabsorption, and malnutrition leading to poor growth, cognitive development and predispose the vulnerable population to additional infectious agents including parasites[12].

Gastrointestinal parasitic infection has been significantly reduced in developed countries by improving sanitation and other hygienic measures including hand washing and adopting proper hand hygiene practices[13]. However, people living in low socioeconomic areas of the society in developing countries suffer from infections of various types of parasites. Although various vehicles (*e.g.*, food, water, feces, *etc.*) of transmission of enteric parasites from person to person have been reported, it remained to be demonstrated that hands of infected persons carry enteric parasites and are potential vehicle of their transmission. The scientific evidence describing intestinal parasites and bacterial contamination on paper currency from a developing country highlight the role of poor hand hygiene practices allowing dissemination of infectious diseases including enteric parasitic ova/(oo)cysts[11]. To our knowledge, this is the first report where enteric parasitic ova/(oo)cysts have been recovered from naturally contaminated hands of populations living in low socioeconomic communities of Indian Subcontinent (Bangladesh and India).

MATERIALS AND METHODS

Selection of human subjects

Human subjects were selected from the low socioeconomic communities of Dhaka, Bangladesh and Kolkata, India, living mainly in the slum area with poor sanitation facility and lack of good hygienic practices (*e.g.*, use of soap for hand washing after defecation; use of toilet tissue after defecation). A total of 300 volunteers from Kolkata, India and 672 volunteers from Dhaka, Bangladesh were selected in this study between April 2009 and June 2010. Volunteers for collection of stool and hand wash samples were selected independent of gender, age, religion, and race by a door to door visit procedure within the selected study region.

Sample preparation for microscopic analysis

Hands of the individual human subjects were washed with 100 mL of phosphate buffered saline (PBS) with rubbing. Total hand wash in PBS (100 mL) was centrifuged at 3000 rpm for 10 min to concentrate enteric parasitic ova/(oo)cysts. Hand wash samples collected from from both sites surveyed, were concentrated using the method of Ridley[14]. The concentrated ova/(oo)cysts thus obtained were suspended in 300 μL PBS. The concentrated ova/(oo)cysts were aliquoted into three parts: 100 μL was used for DNA isolation, 100 μL was used for microscopic analysis and 100 μL was stored at -80 ℃ for additional testing if required.

Microscopic analysis of stool and hand wash samples

Stool samples were tested for the presence of enteric parasitic ova/(oo)cysts using the methods published elsewhere[15]. In brief, a smear of feces in 0.9% saline was examined microscopically for the presence of enteric parasitic ova/(oo)cysts [*Entamoeba histolytica (E. histotylica)*, *Entamoeba dispar (E. dispar)*, *Giardia lamblia (G. lamblia)*, *Iodamoeba butschlii (I. butschlii)*, *Hymenolepis nana* (*H. nana*), *Trichuris trichiura* (*T. trichiura*), *hookworm*, *Cryptosporidium parvum (C. parvum)*, *Trichomonas hominis* (*T. hominis*), *Schistosoma*, *Blastocystis hominis (B. hominis)*, *Ascaris*, and *Taenia*]. Concentrated hand wash samples were directly observed under light microscope. Three separate techniques were used to identify the parasites in fecal samples: iodine wet mount

Table 1 Polymerase chain reaction primers used in this study

Target parasite	Target gene	Primer	Primer sequence (5'-3')	Annealing temperature	PCR Product size (bp)	Ref
Giardia lamblia	Beta-giardin	MAH433F MAH592R	CATAACGACGCCATCGCGGCTCTCAGGAA TTTGTGAGCGCTTCTGTCGTGGCAGCGCTAA	60	218	Rochelle *et al*[19]
Ascaris lumbricoides	rDNA	ITS-1F ITS-1R	TGCACATAAGTACTATTTGCGCGTAT TGATGTAATAGCAGTCGGCGG	60	82	Pecson *et al*[20]
Entamoeba histolytica	SSU rRNA	EH1 EH2	GTACAAAATGGCCAATTCATTCAATG ACTACCAACTGATTGATAGATCAG	51	128	Gonin *et al*[21]
Cryptosporidium sp.	SSU rRNA	18 SF 18 SR	TTCTAGAGCTAATACATGCG CCCTAATCCTTCGAAACAGGA	55	1325	Xiao *et al*[32]
Cryptosporidium sp.	Nested PCR for SSU rRNA		GAAGGGTTGTATTTATTAGATAAAG AAGGAGTAAGGAACAACCTCCA	55	825	Xiao *et al*[32]

PCR: Polymerase chain reaction.

staining for all parasites and parasitic ova/(oo)cysts[16]; modified Kinyoun's Acid fast staining for *Cryptosporidium sp.*[17] and Trichrome staining for *Giardia sp.* and *Entamoeba sp.*[16].

Polymerase chain reaction

Genomic DNA was isolated from stool samples according to the protocol described previously[18]. From handwash samples, total DNA was isolated by using DNA isolation kits (Invitrogen Life Technologies, Carlsbad, CA) according to the instructions provided by the manufacturer. All these DNA samples were used for the identification of enteric parasites present in the sample by polymerase chain reaction (PCR). Parasite-specific primers used in this study[19-21], their annealing temperature and respective PCR product sizes are listed in Table 1. PCR was performed according to the manufacturer's (Invitrogen Life Technologies, Carlsbad, CA) instruction. PCR product (DNA) was characterized by agarose gel electrophoresis. DNA was stained with ethidium bromide, visualized under UV light and images were recorded.

Ethical approval

Each survey site had their protocols reviewed and approved by their respective Institutional Review Boards (IRBs) prior to initiating the survey.

Genotyping of enteric parasites isolated from both stool and handwash samples

DNA was isolated from stool and hand wash samples of individuals, whose both hand wash and stool samples were positive for parasitic ova/(oo)cysts by microscopy. Every 15th Kolkata, India and every 10th Dhaka, Bangladesh handwash positive individual were analyzed this way. Segments of DNA known to be unique to each strain of enteric parasite were amplified by using specific primer sets (Table 1) for PCR. The PCR amplicons were purified with the GFXTM PCR DNA and gel band purification kit (Amersham Pharmacia, United States), and sequenced using the dideoxy-nucleotide chain termination method with the ABI PRISM® BigDye Terminator Cycle Sequencing Reaction kit (Perkin-Elmer Applied Biosystems, Foster, CA) on an automated sequencer (ABI PRISM™ 310). The chromatogram sequencing files were inspected using Chromas 2.23 (Technelysium, Queensland, Australia). Sequence alignments were developed using CLUSTALX 1.81[22].

RESULTS

Recovery and identification of the types of enteric parasitic ova/(oo)cysts in stool samples

To better understand the effect of enteric parasitic burden on human health, we randomly surveyed a group of people living in low socioeconomic areas located in Kolkata, India and in Dhaka, Bangladesh. We found that 38% of the people in Kolkata and 40% in Dhaka were infected with enteric parasites. The different types of parasites detected by microscopy (morphology) are shown in Table 2. We also used PCR for DNA analysis to confirm the identity of these parasites (Figure 1). *A. lumbricoides* infection was the most prevalent (43% in Kolkata and 37% in Dhaka) followed by *Giardia* (26% in Kolkata and 10% in Dhaka) and 20% *Trichuris* in Dhaka. *Taenia* and *H. nana* infections were minimal (2%) in Kolkata and *T. hominis* and hookworm (0.5%) in Dhaka (Table 2). We did not find any pinworm in both study sites, most possibly due to the requirement of specific isolation protocol. These results indicate that a high percentage of people are infected with enteric parasites in both communities surveyed.

Recovery and identification of the types of enteric parasitic ova/(oo)cysts in hand wash samples

To determine if hands become contaminated in people infected with enteric parasites from their stool samples, handwash samples were examined for the presence of enteric parasitic ova/(oo)cysts. It was found that hands from 14% of the people in Kolkata, India and 7% in Dhaka, Bangladesh (Figure 2) that were contaminated with enteric parasites were also infected [stool-positive for enteric parasitic ova/oo(cysts)] with these enteric parasites in their gastrointestinal tract. Further analysis of these parasites by combined microscopy (Table 3),

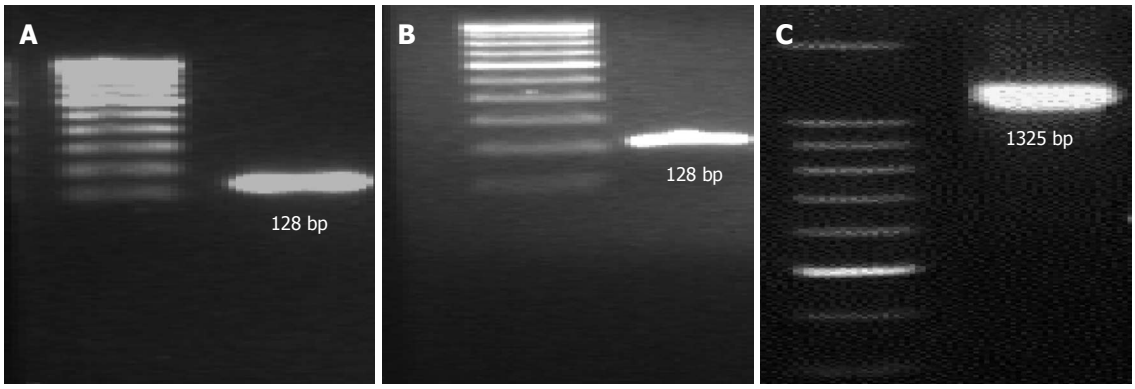

Figure 1 Identification of enteric parasites by polymerase chain reaction. Polymerase chain reaction (PCR) amplification of DNA sequences from stool and handwash samples containing. A: *Entamoeba histolytica*; B: *Giardia lamblia*; C: *Cryptosporidium sp.* Lane M: 1 kb DNA (Invitrogen), Lane E: 128 bp PCR product; Lane E: 128 bp PCR product; Lane C: 1325 bp PCR product ova/(oo)cysts.

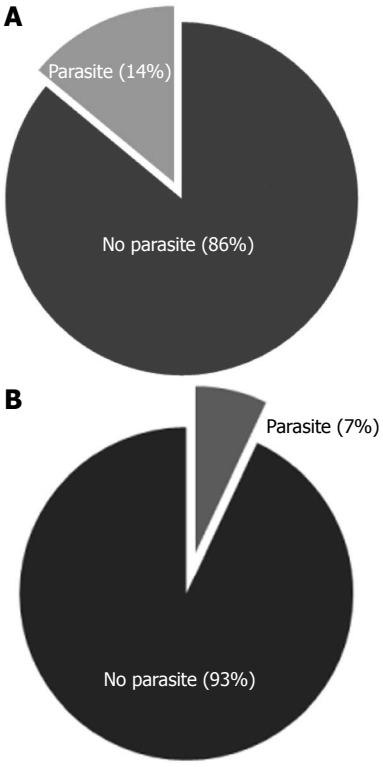

Figure 2 Identification of enteric parasites in hand wash. Hands of the individual human subject were washed with 100 mL of phosphate buffered saline (PBS) with rubbing several times. Total hand wash in PBS was centrifuged to concentrate enteric parasitic ova/(oo)cysts as described in the "Materials and Methods". Concentrated ova/(oo)cysts were studied by microscopic analysis and polymerase chain reaction (using genomic DNA). Presence of ova/(oo)cysts in hand wash samples from Kolkata, India (A) and Dhaka, Bangladesh (B) is shown in percentage.

PCR and permanent staining techniques revealed that *Ascaris* contamination was the most prevalent (53% in Kolkata and 47% in Dhaka) followed by *Giardia* (31% in Kolkata and 19% in Dhaka).

Genotyping of enteric parasitic ova/(oo)cysts isolated from stool and hand wash samples

In order to determine similarity between enteric parasitic ova/oo(cysts) isolated from stool samples and corresponding hands of volunteers, we genotyped these parasitic ova/(oo)cysts obtained from both stool and hand wash samples from the same person by nucleotide sequencing. It was found that PCR products obtained from stool and hand wash samples of each infected person were identical (Table 4). These results confirmed that hands of infected persons were contaminated with corresponding enteric parasite(s) present in their gastrointestinal tract.

Table 2 Characterization of enteric parasitic ova/(oo)cysts recovered from stool samples

Parasites	Prevalence in parasitic ova/(oo)cyst positive stool samples	
	Kolkata, India (n = 113)	Dhaka, Bangladesh (n = 269)
Intestinal protozoa		
Giardia lamblia	26%	10%
Cryptosporidium hominis.	10%	ND
Entamoeba histolytica	ND	7%
Blastocystis hominis	ND	7%
Iodamoeba butschlii	ND	6%
Trichomonas hominus	ND	0.50%
Soil-transmitted helminthes and schistosomes		
Ascaris lumbricoides	43%	37%
Trichuris trichiura	ND	20%
Hookworm	12%	0.50%
Hymenolepis nana	2%	1%
Taenia sp.	2%	ND
Schistosoma sp.	5%	ND

Percentage of each type of parasite found in stool samples from Kolkata, India and Dhaka, Bangladesh. ND: Not detected.

DISCUSSION

It is widely believed that hands are potential vehicle for transmission of infectious agents including enteric parasitic infection. To the best of our knowledge, no experimental evidence supporting this hypothesis regarding role of contaminated hands in dissemination of enteric parasites is available in scientific literature. In this multisite study, we surveyed intestinal parasitic infections in

Table 3 Identification of enteric parasites recovered from hand wash samples

Parasites	Prevalence of enteric parasitic ova /(oo)cysts in hand wash samples								
	Kolkata, India (*n* = 100)	Sex		Age (yr)		Dhaka, Bangladesh (*n* = 100)	Sex		Age (yr)	
		M	F	≤ 12	> 12		M	F	≤ 12	> 12
Intestinal protozoa										
Giardia lamblia	31%	64.5%	35.50%	35.50%	64.5%	19%	86%	14%	100%	0%
Cryptosporidium hominis.	5%	60%	40%	0%	100%	0%	0%	0%	0%	0%
Blastocystis hominis	ND	0%	0%	0%	0%	5%	0%	100%	0%	100%
Iodamoeba butschlii	ND	0%	0%	0%	0%	5%	0%	100%	0%	100%
Soil-transmitted helminthes and schistosomes										
Ascaris lumbricoides	53%	47.10%	52.90%	56.60%	43.40%	47%	68%	32%	100%	0%
Trichuris trichiura	ND	0%	0	0%	0%	24%	100%	0%	100%	0%
Hookworm	9%	44.40%	56.60%	0%	100%	0%	0%	0%	0%	0%
Schistosoma sp.	2%	0%	100%	100%	0%	0%	0%	0%	0%	0%

A portion of concentrated samples were smeared on microscopic slides and examined microscopically and/or by polymerase chain reaction. Percentages of different types of enteric parasites found in hand wash samples are shown. ND: Not detected. M: Male; F: Female.

Table 4 Genotyping of enteric parasites isolated from stool and hand wash samples by DNA sequencing

Enteric parasite studied	Number of samples studied	Number of samples genotyped with 100% similarity	
		Stool	Hand wash
Giardia lamblia	3	3	3
Entamoeba Histolytica	3	3	3
Trichuris trichiura	3	3	3
Giardia lamblia	5	5	5
Ascaris sp.	5	5	5
Trichuris trichiura	5	5	5

Similarities of enteric parasites (*Giardia lamblia*, *Cryptosporidium sp.*, *Ascaris lumbricoids*, and *Entamoeba histolytica*) found among the stool and hand wash samples collected from same individuals.

people from low socioeconomic communities in Bangladesh and India. We report presence of enteric parasitic ova/(oo)cysts on hands of people infected with parasite in their intestinal tracts and shedding their ova/(oo)cysts in stools. These results highlight the role of naturally contaminated hands with enteric parasitic ova/(oo)cysts in dissemination of enteric parasites in the communities with compromised hand hygiene and general hygiene practices.

In an analysis of randomly collected stool samples, we found that about 40% of human populations surveyed were infected with various enteric parasites. Our surveyed population in Dhaka, Bangladesh revealed that 7% of those volunteers infected with enteric parasites had their hands contaminated with parasitic ova/(oo)cysts. In a similar survey conducted in Kolkata, India we found about 14% of infected individuals had their hands contaminated with enteric parasitic ova/(oo)cysts. Difference in percentage of hand contamination of enteric parasites in two sites highlights the importance of analysis of increased sample size and number of sites to identify the cause of variation which could be one of the reasons accounting the for difference in two sites surveyed. Given, our study on contamination of hands with enteric parasitic ova/(oo)cysts was a snapshot in time, it is not known if the actual percentage of hand contamination with enteric parasites is more or less than what is being reported in this study. However, to the best of our knowledge, this is the first report demonstrating natural contamination of hands of human population (children and adults) infected with enteric parasitic ova/(oo)cysts which has also been confirmed by genotypic analysis to be the same parasites recovered from their stool samples.

Studies have shown that asymptomatic enteric infections (such as with *Cryptosporidium*, enteroaggregative *Escherichia coli*, and *Giardia*) are associated with retarded physical and cognitive development[23]. We have found a number of children stool-posive for enteric parasites, had their hands contaminated with these parasitic ova/(oo)cysts. This indicates a possible mechanism of transmission by self-inoculation of parasites in children maintaining the vicious cycle of enteric parasitic chain of infection that may lead to long term effect on their physical and cognitive development.

Hands contaminated with enteric parasitic ova/(oo)cysts can be a potential source of enteric parasitic infections in these communities and highlights the role proper hand hygiene practices could possibly have in reducing parasitic infection in these communities living in developing nations. People living in these communities are involved in working in food shops and other settings (*e.g.*, schools, hospitals and service industries). It appears

that natural contamination of hands of infected people could be a potential source of transmission of all enteric parasites in these hygienically-compromised communities. However, transmission of *Ascaris* through hand contamination remains unclear since *Ascaris* is known to require a contact of soil for hatching their eggs and they could have acquired *Ascaris* ova from the contaminated environment. Further studies are required to determine contribution of hand contamination in transmission of *Ascaris* in the community. In one study, school children from a slum in Visakhapatnam, south India were surveyed for intestinal parasitic load, found the prevalence rate for *A. lumbricoides* was 73%-75% followed by *T. trichiura* (66%) and hookworm (9%)[24,25]. Interestingly, re-infection prevalence post-treatment with albendazole reached pre-intervention level over a nine month period[26]. This highlights the potential role of hygiene to sustain the chemotherapeutic interventions programs designed for prevention and control these enteric parasites[3]. The problem is compounded by the fact that according to UNICEF and World Health Organization's (WHO) estimates, 1.1 billion people lacking safe water (1 in 6 people, or 18% of the world's 2005 population, projected to increase to 2.9 billion by 2025) and 2.4 billion lacking even pit latrines/adequate sanitation (4 in 10, or 42% of people, projected to be 4.2 billion by 2025)[27], consequently affecting adversely on the personal, domestic and community hygiene. Adopting holistic intervention approaches including improved hygiene, clean water supply along with nutrient supplement and chemotherapeutic intervention for combating infectious diseases including enteric parasites can potentially contribute not only to child's growth and cognitive development but also economic prosperity of the target population as experienced by developed nations. It is interesting to note that in emerging market such as India, the populations in general have more access to cell phones than toilets (http://www.inweh.unu.edu/News/2010-04_UNU-INWEH_News Release_Sanitation.pdf). Globally, roughly 1.5 billion individuals are infected with one of these parasites, *Ascaris*, primarily in Africa and Asia. In this regard, the developed nations are not fully immune to enteric parasites. Ascariasis is endemic in the United States as well. One study found that the prevalence of Ascariasis in the United States at about 4 million[28]. In a survey of a rural Nova Scotia (Canada) community, 28.1% of 431 individuals tested were positive for Ascaris, all of them being under age 20, while all 276 tested in metropolitan Halifax were negative[29] indicating disparity even within developed nations.

Therefore, according to UNICEF the role of water, sanitation, and hygiene (WASH) is critical for sustainable development contributing to the U.N.'s Millennium Development Goals which is to provide water and sanitation to fifty percent of population without access to safe water and basic sanitation, by 2015 (http://www.un.org/millenniumgoals/environ.shtml). Currently the UNICEF is promoting "WASH in schools to improve health" by lessening the spread of infectious diseases. Therefore, the potential mitigational role of hygiene in prevention of infectious agents including enteric parasites and thereby contributing to the child's physical and cognitive development cannot be under estimated[30]. According to WHO list of neglected tropical diseases (NTDs) (http://www.who.int/neglected_diseases/diseases/en/), the intestinal protozoa, STHs and schistosomes recovered from naturally contaminated hands of population surveyed, are amongst the main NTDs which can be prevented by adopting holistic approach including proper sanitation and hygiene measures[3,12].

In previous studies, bacteria/viruses have been reported to be present on hands and suggested to play an important role in their transmission in community[31]. In this study we report contamination of human hands with enteric parasites in two independent sites surveyed in two developing countries of the Indian Subcontinent. Our study indicates that contamination of hands with parasite ova/(oo)cysts are common among those populations already infected and this may play a role in continued cycle of transmission and/or re-infection within the community. Therefore, in addition to chemotherapeutic interventions, food and water sanitization, and regular hand hygiene practices would play a major role in reducing enteric parasitic infections. It will be useful to investigate the effectiveness of hand hygiene products (soap/hand wash agents) in removing enteric parasitic ova/(oo)cysts from naturally contaminated hands in these communities.

COMMENTS

Background

Gastrointestinal parasitic infection has been significantly reduced in developed countries by improving sanitation and other hygienic measures including hand washing and adopting proper hand hygiene practices. However, people living in low socioeconomic areas of the society in developing countries suffer from infections of various types of enteric parasites.

Research frontiers

Studies have shown that asymptomatic enteric infections (such as with *Cryptosporidium*, enteroaggregative *Escherichia coli*, and *Giardia*) are associated with retarded physical and cognitive development.

Innovations and breakthroughs

The results highlight the role of naturally contaminated hands with enteric parasitic ova/(oo)cysts in dissemination of enteric parasites in the communities with compromised hand hygiene and general hygiene practices.

Terminology

This study indicates that contamination of hands with parasite ova/(oo)cysts are common among those populations already infected and this may play a role in continued cycle of transmission and/or re-infection within the community.

Peer review

This is a well written manuscript that clearly demonstrates contamination of human hands with enteric parasites present in the gastrointestinal tract of the tested individuals themselves. The study is based on two independent sites surveyed in two developing countries, and the results found were very similar for both sites. The study demonstrates that the contamination of hands with parasite ova/(oo)cysts are common among low income populations infected with enteric parasites and indicate that this may play a role in continued cycle of transmission and/or re-infection within the community. This manuscript strengthens the importance of hand hygiene in reducing the spread of parasites in particular and infectious diseases in general.

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CASE REPORT

Liver biopsy for visceral leishmaniasis diagnosis in pregnancy: report of 2 cases

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Abstract

Visceral leishmaniasis (VL) or kala-azar is a zoonosis caused by intracellular protozoa of the *Leishmania* genus and is transmitted to humans by the bite of phlebotomine sandflies. It particularly affects cells in the phagocytic mononuclear system, accompanied by disturbances of cellular and humoral immunity. VL is potentially fatal and is characterized by fever, hepatosplenomegaly, diarrhea, epistaxis, jaundice, anemia, leucopenia, thrombocytopenia, hypoalbuminemia and hyperglobulinemia. Diagnostic suspicion is based on epidemiological, clinical and laboratory data and is confirmed by detecting the parasite in infected tissue. Splenic aspiration is the most sensitive method, followed by bone marrow aspiration (BMA) by sternal puncture, liver biopsy and lymph node aspiration; but, due to safety concerns, BMA is the most recommended method. VL is included as a target disease by players in drug research and development. Severe liver dysfunction associated with VL is uncommon. We report two VL cases in pregnant women from Bauru, Sao Paulo state, Brazil, considered an endemic area. The first of them developed hepatic failure due to fulminant hepatitis. In both cases, BMA was unable to find the protozoan; thus, liver biopsy was the only means of making the diagnosis.

Key words: Visceral Leishmaniasis; Infection in pregnancy; Liver biopsy; Bone marrow aspirate; Differential diagnosis

Core tip: Visceral leishmaniasis, which has many severe presentations, is an endemic disease found in many countries around the world, especially in South America, where Brazil is the most affected country. We herein present two cases of this disease affecting women during pregnancy, when the diagnosis and management can be very difficult. In both patients, the usual method of diagnosis failed so liver biopsy was the only option to make the correct diagnosis. Therefore, liver biopsy may be considered in special situations when severe visceral leishmaniasis is suspected, as in the cases herein presented.

Lima TB, Villar CR, Rodrigues MAM, Baima JP, Yamashiro FS, Franzoni LC, Caramori CA, Silva GF, Romeiro FG, Sassaki LY. Liver biopsy for visceral leishmaniasis diagnosis in

pregnancy: report of 2 cases. *World J Clin Infect Dis* 2013; 3(2): 20-24 Available from: URL: http://www.wjgnet.com/2220-3176/full/v3/i2/20.htm DOI: http://dx.doi.org/10.5495/wjcid.v3.i2.20

INTRODUCTION

Visceral leishmaniasis (VL) or kala-azar is a zoonosis caused by intracellular protozoa of the Leishmania genus. The disease is transmitted to humans by the bite of phlebotomine sandflies and Lutzomyia longipalis is one of the major transmitting agents. In Latin America, 90% of VL cases occur in Brazil, where the disease is found in 19 states and affects approximately 1600 cities[1,2]. The main reservoirs in wild and household environments are foxes and dogs, respectively[1,2]. The disease affects the phagocytic mononuclear system, leading to disturbances of cellular and humoral immunity[3,4]. The city of Bauru, 325 km from the state capital, is considered an endemic area for VL[5]. The disease affects individuals at any age and children under 10 years are commonly involved in endemic areas[6,7]. In contrast, VL is rare among pregnant women and there are few studies in these patients. The most common manifestations are fever, hepatosplenomegaly, diarrhea, epistaxis, jaundice, anemia, leucopenia, thrombocytopenia, hypoalbuminemia and hyperglobulinemia[1]. Diagnostic suspicion is based on epidemiological, clinical and laboratory data. In endemic regions, the diagnosis is confirmed by detecting the parasite in infected tissue and the first recommended option is bone marrow aspiration (BMA) by sternal puncture. Given that gestational VL is rare in South America, severe liver dysfunction associated with this diagnosis is uncommon[1]. Herein, we report two VL cases in pregnant women from Bauru, Sao Paulo state, Brazil, the first of which developed fulminant hepatic failure. In both cases, BMA was negative and liver biopsy was necessary to make the diagnosis.

CASE REPORT

Case 1

A 26 year old pregnant woman had a preterm vaginal delivery after 5 d of daily fever associated with hypogastric abdominal pain and foul lochia. She was initially treated with cephalothin and later with ampicillin, amikacin and metronidazole, prescribed due to suspected puerperal infection. She developed jaundice, choluria, hepatosplenomegaly and increased liver enzymes (Table 1). Imipenem and vancomycin were then introduced. Abdominal ultrasonography (US) and computed tomography showed no alterations in the liver or biliary tract. Transvaginal ultrasonography also showed no alterations. Serology for viral hepatitis, autoantibodies, ceruloplasmin, iron profiles, blood cultures and urocultures were negative. The patient had pancytopenia which was investigated by BMA but no specific alterations were found. Due to progressive liver failure, percutaneous liver biopsy was performed and the liver histology showed acute hepatitis, with intense mixed portal inflammation and structures compatible with amastigotes (Figure 1). The patient was treated immediately with liposomal amphotericin B, at a total dose of 20 mg/kg body weight, for 5 d. At that time, anemia, thrombocytopenia and abdominal pain worsened. Subsequent US identified a clot retained in the abdomen which was removed by exploratory laparotomy. Hemotherapeutic support was then initiated, including concentrations of red blood cells and platelets, as well as fresh plasma, cryoprecipitates and neutrophil colony-stimulating factors. The patient became unconscious, with increased serum ammonia and bilirubin levels concomitant with a progressive reduction in aminotransferases, thus characterizing hepatic failure due to fulminant hepatitis. Despite all efforts, she died on the 32nd puerperal day.

Case 2

A 31 year old woman in the 12th week of pregnancy arrived at the hospital with epigastric and right flank pain, progressive jaundice and choluria that had persisted for the previous 12 d. Physical examination showed jaundice without visceromegaly or fever. Laboratory tests suggested cholestasis and hepatocellular lesions. Serology for viral hepatitis was negative and no signs of biliary obstruction were found at abdominal US. BMA was performed but no specific findings were observed. Percutaneous liver biopsy was indicated and the histological analysis showed acute hepatitis and the presence of amastigotes, compatible with VL. Liposomal amphotericin B infusions were immediately initiated (at the 12nd gestational week) and this time the treatment was followed by clinical and laboratory improvement (Table 2).

DISCUSSION

There are no estimates of VL in pregnant women, particularly due to the small number of publications on such cases. In South America, VL in pregnant women is considered to be rare and the first Brazilian case was reported in 1993[8,9]. In case 1, clinical, laboratory and epidemiological data led to suspicion of VL because the patient had been residing in an endemic city (Bauru) and presented with associated symptoms of daily fever and acute hepatitis. Despite the possibility of trans-infectious hepatitis, the major causes of acute hepatitis were investigated, including viral diseases (hepatitis A, B and C, herpes simplex, cytomegalovirus, varicella, dengue fever, Epstein-Barr), which represent 40% of jaundice causes in pregnancy. These viral diseases do not usually affect the natural course of VL, except for hepatitis E and herpes simplex virus (HSV), which may lead to acute liver failure and fetal loss. Although HSV is considered to be a rare hepatitis agent, it can cause severe hepatitis in immunosuppressed individuals, neonates, pregnant women and transplant patients, with a mortality rate of up to 50% or even 60%[10,11]. The patient showed no

Table 1 Biochemical test profile, blood and coagulation tests of the 1st case

	Post-partum (d)				
	19	24	29	32	Normal range
Biochemical test profile					
GGT (U/L)	577	289	47	58	15-73
Alkaline Phosphatase (U/L)	1081	924	121	103	36-126
Aspartate transaminase (U/L)	338	3879	1212	429	30-110
Alanine transferase (U/L)	94	727	279	110	21-75
Albumin (g/dL)	2.3	2	1	1.5	3.5-5
INR	1.34	2.55	2.51	1.68	< 1.25
Total bilirubin (mg/dL)	13	15	10	16	0.2-1.3
Indirect bilirubin (mg/dL)	3	4	2	2	0-1.1
Direct bilirubin (mg/dL)	10	11	8	14	0-0.3
Blood and coagulation tests					
Platelets (× 10^3/mm^3)	155	132	55	22	140-440
Leukocytes (× 10^3/mm^3)	7.3	5	2.5	0.9	4-11
Hemoglobin (g/dL)	10	9.7	4.4	10.5	12-16
Factor Ⅴ (%)	-	70.2	40.7	64.8	50-150
Factor Ⅶ (%)	-	43.5	36	35.2	50-150
Fibrinogen (mg/dL)	-	164	90	193	146-380

GGT: Gamma-glutamyl transpeptidase; INR: International normalized ratio.

Table 2 Main laboratory tests of the 2nd case during LV treatment

	Gestational week				
	12	13	14	22	Normal range
GGT (U/L)	102	94	83	9	15-73
Alkaline Phosphatase (U/L)	116	106	109	45	36-126
Aspartate transaminase (U/L)	1711	2041	1905	16	30-110
Alanine transferase (U/L)	2198	2237	1809	15	21-75
Albumin (g/dL)	3.3	3.4	3.5	-	3.5-5
INR	1.29	1.51	1.69	1.2	< 1.25
Total bilirubin (mg/dL)	14.5	19.6	24.6	0.6	0.2-1.3
Indirect bilirubin (mg/dL)	1.6	2	2.4	0.4	0-1.1
Direct bilirubin (mg/dL)	10.7	15	16.3	0	0-0.3

GGT: Gamma-glutamyl transpeptidase; INR: International normalized ratio. LV treatment was initiated at the 12th gestational week.

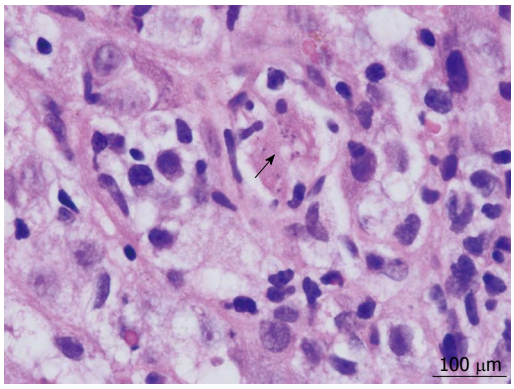

Figure 1 Pathological findings (hematoxylin/eosin staining × 1000 magnification-immersion oil). Liver biopsy. Structures compatible with amastigotes (arrow).

improvement after the use of broad spectrum antibiotics. Therefore, other possible hepatic diseases were also investigated: autoimmune hepatitis (AIH), Wilson's disease, as well as other lymphoproliferative, metastatic and metabolic diseases. With regards to the liver diseases associated with pregnancy, the most common are viral and drug-induced hepatitis, pre-eclampsia, acute hepatic steatosis in pregnancy (AHSP) and intrahepatic cholestasis in pregnancy (IHCP)[11-14]. The HELLP Syndrome (Hemolysis, Elevated Liver enzymes and Low Platelets) is a serious complication that most frequently occurs in the 3rd trimester but can also occur in the puerperium in 25% of cases[15]. However, the first case had no signs of hemolysis or the presence of schizocytes in the peripheral blood. AHSP occurs between the 30th and 40th pregnancy weeks and begins with the slow onset of anorexia, indisposition and cephalea, followed by vomiting, abdominal pain, fever and, at a later phase, jaundice[16-18]. In 2% of cases, AHSP causes acute hepatic failure, requiring urgent liver transplantation[19]. However, patients with AHSP tend to show clinical and laboratory

improvement after delivery, which did not happen in the case described. IHCP manifests in the 3rd gestational trimester with jaundice and pruritus in up to 70% of cases, but liver function deterioration is rare[12,18,20-22]. AIH affects young patients and due to the conditions of physiological immunosuppression induced by pregnancy, the activity of the disease may be exacerbated after delivery when immunological conditions are reversed. Again, case 1 did not meet the criteria established by the International AIH Study Group[17] so a diagnosis of AIH was rejected. When VL became the major diagnostic suspicion, other exams were considered. Splenic aspiration is the most sensitive method (90%-95%), followed by BMA (80%-90%), liver biopsy and lymph node aspiration, but BMA is the method recommended due to safety concerns[23]. When BMA is performed, the absence of leishmania in BMA does not preclude a diagnosis of the disease. For this reason, percutaneous liver biopsy was indicated and confirmed the diagnosis. Liver compromise resulting from VL has been reported in approximately 2% to 28% of cases[24]. Hepatomegaly may occur in up to 90% of cases, followed by slight increase in liver enzymes without severe disorders[25]. The presence of the parasites in Kupffer cells may be found in up to 40% of cases before treatment[26]. Severe cases with a bad prognosis have been associated with severe anemia, fever for longer than 60 d, diarrhea and jaundice[27]. Fulminant liver failure (FLF), which rarely occurs in VL, has been described more often in children[1]. According to a previous study by Singh *et al*[28] of 155 VL cases with liver compromise, moderate liver dysfunction was found in 16% and FLF in 1.6% of cases. Malatesha *et al*[29] reported an isolated case of an immunocompetent adult male with FLF by VL who recovered after therapy with amphotericin B. Unfortunately, our first case developed FLF without any response to liposomal amphotericin B. During the hospitalization, she developed multiple organ dysfunction and her 7 year old daughter was also hospitalized with fever and VL, which was successfully treated. If liver biopsy had not been performed to confirm the maternal disease, the child would not have been diagnosed so early. The rapid diagnosis was critical to the successful treatment of this child. Our second case was an oligosymptomatic (without fever or splenomegaly) pregnant woman. The lack of fever and visceromegaly could have delayed the diagnosis but the positive VL epidemiology was fundamental for the diagnostic suspicion. Once again, BMA was not confirmatory. The progressive increase in transaminases and liver dysfunction were the criteria used to indicate the liver biopsy. The American Association for the Study of Liver Diseases does not consider pregnancy to be a contraindication to this procedure[30]. Therefore, a diagnosis of VL was confirmed by the liver biopsy, allowing immediate treatment and resulting in the favorable outcome in this second case. Serological tests may be particularly useful for diagnosing VL given their high predictive value in the diagnosis of immunocompetent individuals. However, in severe cases from endemic areas, they are not sufficient to indicate the specific therapy, because the time required for cured individuals to return to a negative serology (anti-leishmania) is not known. Data suggest that a cellular immune response may still be present in subjects cured of the disease. This would explain the persistence of significant *Leishmania sp.* antibody titers in some subjects after treatment[31]. Thus, a positive test in the absence of clinical manifestations does not authorize the administration of therapy, which is not free of toxic effects.

VL has epidemiological importance in South America, especially in Brazil. Although the disease is rare among pregnant women and rarely causes severe liver dysfunction, both situations can be present in the same patient, requiring early and accurate diagnosis to reduce morbidity and mortality rates. The contribution of liver biopsy as an alternative to BMA in parasite detection was noteworthy in our cases. We conclude that other patients with VL in whom BMA is negative can obtain a correct diagnosis if liver biopsy is performed, as we showed in our last case.

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REVIEW

Acinetobacter baumannii: An emerging pathogenic threat to public health

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Abstract

Over the last three decades, *Acinetobacter* has gained importance as a leading nosocomial pathogen, partly due to its impressive genetic capabilities to acquire resistance and partly due to high selective pressure, especially in critical care units. This low-virulence organism has turned into a multidrug resistant pathogen and now alarming healthcare providers worldwide. *Acinetobacter baumannii* (*A. baumannii*) is a major species, contributing about 80% of all *Acinetobacter* hospital-acquired infections. It disseminates antibiotic resistance by virtue of its extraordinary ability to accept or donate resistance plasmids. The procedures for breaking the route of transmission are still proper hand washing and personal hygiene (both the patient and the healthcare professional), reducing patient's biofilm burden from skin, and judicious use of antimicrobial agents. The increasing incidence of extended-spectrum beta-lactamases and carbapenemases in *A. baumannii* leaves almost no cure for these "bad bugs". To control hospital outbreaks of multidrug resistant-*Acinetobacter* infection, we need to contain their dissemination or require new drugs or a rational combination therapy. The optimal treatment for multidrug-resistant *A. baumannii* infection has not been clearly established, and empirical therapy continues to require knowledge of susceptibility patterns of isolates from one's own institution. This review mainly focused on general features and introduction to *A. baumannii* and its epidemiological status, potential sources of infection, risk factors, and strategies to control infection to minimize spread.

Key words: *Acinetobacter*; *Acinetobacter baumannii*; Biofilm; Combination therapy; Hospital-acquired infection; Intensive care unit; Multidrug resistance; Nosocomial Pathogen; Risk factor

Core tip: *Acinetobacter*, is Gram-negative cocco-bacilli, originally regarded as low virulence bacteria, adopted now with increasing incidences, and recognized as a significant healthcare-associated multidrug-resistant classical pathogen. *Acinetobacter baumannii* (*A. baumannii*) accounts for nearly 80% of reported *Acinetobacter* infections. *A. baumannii* resist desiccation, and survive for several months on animate and inanimate surfaces. It has excellent colonizing potential, and contact transmission is a big challenge intermittent as well as endemic outbreaks. Strong biofilm formation is a part of virulence pathogenesis strategies of this organisms, and elimination of the identified source often require multiple interventions. This review mainly discusses on relevant epidemiological features of *A. baumannii*.

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IMPORTANCE

Once documented as a pathogen with low virulence, *Acinetobacter* is currently an important etiological agent of nosocomial infections, including hospital-acquired pneumonia and ventilator-associated pneumonia in patients admitted to intensive care units (ICUs), wound infections from war, and natural disasters such as a tsunami[1-3]. The National Nosocomial Infections Surveillance System reported a significant increase in the proportion of *Acinetobacter* among all Gram-negative aerobes during the 17 years of the study period (1986 through 2003)[2]. *Acinetobacter* was the only pathogen showing consistently increasing incidence in nosocomial pneumonias, and *Acinetobacter baumannii* (*A. baumannii*) was a major species among reported causes of nosocomial pneumonia[3].

Taxonomical aspect

The Gram-negative, non-fermentative aerobic bacteria which now recognized as belonging to the genus *Acinetobacter* have in the past been classified under various generic names. The genus *Acinetobacter* is now classified in the family *Moraxillaceae*, which includes Moraxella, Acinetobacter, Psychrobacter, and related organisms[4]. The genus *Acinetobacter* includes *Gram-negative coccobacilli* that have a G + C content of 39-47 mol% and that are strictly aerobic, non-motile, catalase-positive, and oxidase-negative. The negative oxidase test is important for rapid presumptive identification to differentiate the genus *Acinetobacter* from other similar non-fermentative organisms. But the transformation assay of Juni is the only test considered to be an unambiguous identification test for the genus *Acinetobacter*[5]. Most *Acinetobacter* species are non-fastidious and can be easily grown on simple microbiological media. Although variants appear, typical colonies are smooth, domed shaped pale yellow to grayish, about 2 mm with entire edge. Most species grow at ambient temperature, and pathogenic species such as *A. baumannii* grow well at 37 ℃. Enrichment medium such as Leeds selective medium as occasionally use, and are helpful in recovery of isolates from complex samples[6].

The genus *Acinetobacter* encompasses at least 25 DNA groups (genospecies) identified by DNA-DNA hybridization, 23 of which have been officially validated[7-10]. A recently submitted species of *Acinetobacter nosocomialis* (*A. nosocomialis*) and *A. pittii* are included in taxonomic nomenclature. *Acinetobacter* uses a wide variety of organic compounds as a carbon sources. This property has been used in developing the identification system for this organism. It is often difficult in clinical laboratories to differentiate the isolates of *Acinetobacter* at the species level according to their phenotypic characteristics[8], and can be inadequate for species confirmation, and should be used with caution. Automated systems available for distinguishing Gram-negative pathogens can identify *Acinetobacter* species but have limitations. *A. baumannii*, *Acinetobacter calcoaceticus* (*A. calcoaceticus*), genomic species 3, and 13TU are closely related and formally grouped as *A. baumannii-A. calcoaceticus* (Abc) complex (recently species 3 and 13TU are referred as *A. pittii* and *A. nosocomialis*, respectively). Molecular characterization, particularly 16S rRNA gene sequence analysis, can be of great help to resolve matters of dispute. Looking at the global dissemination of international clones, and their involvement in outbreaks, the rapid and discriminating genotyping methods are required for delineation of such clonal lineages[11]. Among the most common methods that are currently used involves pulse-field gel electrophoresis, amplified fragment length polymorphism, single locus genotyping, trilocus sequence-based typing, multi-locus sequence typing such as PubMLST, Pasteur's MLST, multi-locus variable-number tandem-repeats , resistance island typing, PCR with electrospray ionization mass spectrometry, next-generation whole genome sequencing, and PCR-based replicon of plasmid DNA. Most of these genotyping methods are not routinely used in hospitals and not cost effective, but extremely useful for to establish clonal relationships of the isolates and their taxonomical classification[11-15].

Habitat and colonization

Although *Acinetobacter* has emerged as an important pathogen, little is known about its natural reservoirs and habitat. Pathogenic members of the genus *Acinetobacter* contribute to the normal flora of human skin, upper respiratory tract, and gastrointestinal tract. The clinical consequences of *Acinetobacter* infections range from minimal to moderate to severe. *A. baumannii,* along with two other genetically closely related species (genomic species 3 and 13TU), is almost exclusively associated with human infection and is phenotypically difficult to differentiate routinely in clinical laboratories. Hence, the group is known as *A. baumannii-A. calcoaceticus*-complex (*Abc*-complex), and is often regarded *A. baumannii* in clinical practice as[16,17]. Although many consider *A. baumannii* to be ubiquitous, not everyone agrees. It is considered to be commensal with humans, and colonization is well documented. Therefore, the switch from colonization to infection is more favorable than it would be from more distant environmental sources[10]. Other species that are occasionally isolated from clinical samples are *A. calcoaceticus, A. hemolyticus, Acinetobacter johnsonii* (*A. johnsonii*), *Acinetobacter lowffi*, and *Acinetobacter ursingii*.

EPIDEMIOLOGICAL ASPECT

Acinetobacter species account for a substantial proportion of epidemic and endemic nosocomial infections and occasional sporadic outbreaks[14,16,18,19]. Geographically distant outbreaks are being studied for their ancestral genetic pool and clonal lineage. Multilocus sequence typing analysis recognized Ⅰ to Ⅲ international clones, corresponds to their clonal complexes, and many of the isolates causing outbreaks are suspected phylogenetically to be closely related with these clonal groups[20]. It can cause a wide array of infections such as respiratory tract infections, bloodstream infections, urinary tract infec-

tions, meningitis, endocarditis, and wound infections. In a recent report, 6 out of 7 patients with *Acinetobacter* bloodstream infections found *A. baumannii* colonizing their gastrointestinal tract[21]. *A. baumannii* is a prevalent species that causes epidemic outbreaks of nosocomial *Acinetobacter* infections[17,22-24]. Although there are mixed opinions, *A. baumannii* is usually reported to have a known natural habitat around patient population and in healthcare facilities and is occasionally isolated from environmental samples such as soil and water. *A. baumannii* is an excellent colonizer and is known to form biofilms. Furthermore, the reports demonstrate a positive correlation between biofilm formation capabilities and the multidrug resistance (MDR) status of *A. baumannii*. Such phenotypes have the ability to mediate outbreaks[25]. The multifactorial nature of the pathogenicity of *A. baumannii* has been documented recently and various models are proposed, and the involvement the presence and expression of exoproteases and exopolysaccharides (mediating biofilms), iron acquisition resistance to serum, resistance to desiccation, adherence and colonization, epithelial cell invasion and extraordinary ability to acquire foreign genetic material through lateral transfer for own survival, are elaborated as virulence attributes[26-31]. *A. baumannii* survives for a relatively long time in environments such as dry animate and inanimate surfaces and, when conditions are favorable, leads to outbreaks. The exact natural habitat of many of the *Acinetobacter* species is yet to be fully understood and may require intense efforts to identify.

Towner describes that depending on the site of isolation and the population of species or strains involved, *Acinetobacter* can be broadly categorized into three groups[10]: (1) MDR isolates capable of colonizing and infecting hospitalized patients, usually mediating hospital outbreaks. Generally these are *A. baumannii*. The isolates usually belong to a single clone or limited clones. Intensive care units are the depots for such outbreaks (occasionally other units mediate their spread as well); and (2) Relatively less resistant, less virulent strains that occasionally cause outbreaks. These isolates can be a part of normal skin flora of humans or animals or are associated with food spoilage[10,32]. Examples of such isolates are *A. johnsonii*, *A. lwoffii*, and *A. radioresistens*[33]. Environmental sources of isolate that are sensitive to many routine antibiotics and rarely cause outbreaks. *A. calcoaceticus* is a classic example. Infection control practices are therefore reserved mainly for the resistant isolates, which are usually *A. baumannii*-complex members. The patterns of spread of these members are also peculiar and can be correlated to strains causing outbreaks. In many European and Asian hospitals, the clonal spread (single clone) of *A. baumannii* has been reported either in a single hospital or in multiple hospitals and the strain was susceptible only to colistin and tigecycline[34,35]. Epidemiological typing methods are often helpful in delineating their dissemination and the strains involved in an outbreak and can differentiate epidemic outbreaks from sporadic strains. Thus, overall diversity of habitat, predilection to accumulate antimicrobial resistance, resistance to desiccation, ability to form biofilm, and propensity to cause hospital infection outbreaks make *Acinetobacter* an remarkable microorganism.

A. baumannii strains are generally more resistant than other species of this genus and often express a MDR phenotype, as discussed previously. Therefore, treatment of nosocomial infections caused by *A. baumannii* has become complicated because of the widespread antimicrobial resistance among these organisms[36]. The rising trend of resistance in *A. baumannii* strains, particularly to newer antimicrobial agents, is a health care concern. The organism expresses multiple mechanisms of antibiotic resistance that likely leads to the development of multiply resistant or even "pan-resistant" strains. This situation is particularly a quandary in terms of therapeutic choices for epidemic outbreaks mediated by these phenotypes.

POTENTIAL SOURCES OF INFECTION AND CONTAMINATION

The source of *A. baumannii* infections can be endogenous or exogenous. Most frequently, the infection is exogenous in origin because of the ability of the organisms to survive longer in the environment and on dry surfaces and because they are resistant to desiccation. *A. baumannii* multiply not only on human and animal skin, but also in soil and water and thus have a diversity of reservoirs. Locations in the hospital environment where *A. baumannii* have been found include ventilator tubing, suction catheters, humidifiers, containers of distilled water, urine collection jugs, intravenous nutrition, multidose vials of medication, potable water, moist bedding articles, pillows, and inadequately sterilized reusable arterial pressure transducers[37-39]. *A. baumannii* have been found in or on water taps, sinks, and computer keyboards and on all other inanimate surfaces that can act as a reservoir[40,41]. Hospital food can also be a potential source of *Acinetobacter* infection[8]. A study of two hospital outbreaks in Leiden, the Netherlands, reported the isolation of the outbreak strains from the dust inside the respiratory ventilator, the apparatus used to cool or warm a patient[40].

The gloves, gowns, and unwashed hands of hospital staff including doctors and nurses are frequently contaminated and may act as a potential source of *Acinetobacter* infection[14,42]. Hospital staffs with damaged skin are at increased risk of being colonized with *Acinetobacter* and are more likely to contaminate medical equipment and devices and patients by direct contact, thereby causing outbreaks of infection[43]. Specific types of medical procedures are also reportedly associated with high rates of infection with *Acinetobacter*, such as wound irrigation and treatment, catheterization, and tracheostomy[44]. Thus, the mode of infection can be environmental contamination or cross-contamination[45]. Community-acquired *A. baumannii* pneumonia is one of the severe forms of infection found around Indian Ocean, with very high co-morbidities and

reportedly associated in part with casualties from natural disasters such as earthquake and tsunamis, and wound contamination occurring among soldiers following war-related injuries[46].

NOSOCOMIAL ACQUISITION AND RISK FACTORS

Several factors reported by different groups increase the risk of nosocomial infection with *A. baumannii*. Most vulnerable among them are mechanical ventilation (source of ventilator-associated pneumonia), intensive care and other critical care units, wound and burn units, prolonged hospital stay, prior antibiotic therapy, increased exposures to infected patients, colonized neighboring patients, and health care personnel. Other risk factors are a weakened immune system, chronic and debilitating disease, and diabetes. Infection secondary to an invasive procedure is widely reported and involves ventilator-associated pneumonia, secondary meningitis and bloodstream infection, urinary tract infection, surgical site infection, and catheter-related bloodstream infection. In most cases it is point source contamination. Postoperative complications from infection with *A. baumannii* have been reported; the major risk factors are skin and soft tissue, bone, central nervous system trauma or injuries, and combat wounds and injuries[47-49]. Post-disaster infections caused by *A. baumannii* have also been reported[50,51]. *A. baumannii* is intrinsically resistant to many antimicrobial agents and has a propensity to acquire resistance to other, newer antimicrobial agents as well[52]. Consequently, it has become more prevalent because of selective pressure from antimicrobial agents in ICUs. Analysis of the epidemiological profile of antibiotic-resistant *Acinetobacter* spp showed an increased risk of infection in patients in ICUs who probably spread large numbers of *A. baumannii* cells into their surroundings by shedding *A. baumannii*-infected or colonized cells, making the area more likely to be a source of infection for others[37,53]. Although airborne transmission has been documented, direct contact, including patient-to-patient and health care-provider-to-patient transmission, is more relevant.

Community acquisition of *Acinetobacter* infection, although rare, has been reported[54,55], and a community-acquired MDR *Acinetobacter* carrying IMP1 metallo-β-lactamase, responsible for hospital infection, is recovered[55]. Community-acquired *A. baumannii* pneumonia[56,57], community-acquired bacteremia[58], urinary tract infection[59], and meningitis[54] have been reported. On the basis of the rising incidence of community-acquired *A. baumannii* infection, a concurrent spread of multidrug resistance is the greatest risk. Among, *A. baumannii* wound infections, three hypotheses usually described are a combination of wound with environmental bacteria, a wound contamination from previous cutaneous or oropharyngeal endogenous reservoir, and hospital acquisition[46].

STRATEGIES TO CONTROL INFECTION

Outbreaks, particularly endemic or periodic epidemic outbreaks, caused by MDR *A. baumannii* are difficult to control. It is still possible to effectively control *A. baumannii,* although eradication is in question[14]. Decontamination of the patient by treating the gut and skin has been reported. Antibiotics can be used to inhibit gut colonization by *A. baumannii* that remains susceptible, but the benefits are limited because of the risk of developing resistant phenotypes. Additional research is needed to clarify the role of such techniques for selective decontamination of gut compared with surfaces such as skin[60]. The role of various sites of *A. baumannii* colonization and the risk of epidemiological outbreaks have been assessed; selective gut decontamination was found to be less effective as an additional measure[61]. Selective decontamination of skin with chlorhexidine reduced a significant load of *A. baumannii* and has been proposed as the infection control measure to lower the number of endemic outbreaks[62]. Because *A. baumannii* is widely present in the hospital environment, it can contaminate any surface or article with which it comes in contact, *e.g.*, resuscitation bags, blood pressure cuffs, parenteral fluids and nutritional solutions, lotion dispensers, hand creams, bed linen, and mattresses. Therefore strict hand hygiene and personal cleanliness are essential in breaking the route of transmission[63]. Periodic disinfection of wards, units, and surfaces and sterilization of medical devices using appropriate methods are highly recommended. A periodic hospital environmental sample survey for microbiological contamination is advisable[64,65]. The epidemiological studies help to identify the source or reservoir of the infection and thus eventually to understand how to control the outbreaks[8]. Control of the environmental reservoir is a major part of an effective control strategy[64,66]. The researchers who conducted the study in the Netherlands controlled an outbreak by removing dust from the mechanical ventilator and continuous venovenous hemofiltration machines and replacing dust filters[40]. A study conducted in the United States reported *A. baumannii* as a model in eradication of MDR infections[67]. The control measures for *A. baumannii* infection have been discussed by many investigators[8,18,68,69]. Some of the specific control measures for *A. baumannii* infection are shown in Table 1.

One of the most associated factors with reservoirs is biofilm formation capability of *A. baumannii* wherein it is responsible in part for the intermittent release of pathogens that leads to outbreaks. Biofilm formation by this organism also facilitates its persistence, and thus acts as a source of infection[25]. Recently, a dynamic exchange of gene cassettes between integrons (a mobile genetic element responsible for recruitment of multiple resistance genes, *e.g.,* class 1 integron) in natural biofilms has been demonstrated[25,79]. This association of biofilm is important in higher tolerance or resistance to strong

Table 1 Some of the major infection control measures marked for *Acinetobacter baumannii* infection outbreaks

Sr	Effective control measure	Ref
1	Early detection of a colonized patient or the source or reservoir of an infection	[14,70]
2	Eradication of the source or reservoir	[71]
3	Isolation of an infected or colonized patient into an isolation cubicle	[18]
4	Cohort nursing	[72]
5	Emphasis on hand washing (with alcoholic-based disinfectants) before and after patient handling	[73]
6	Use of disposable gloves and aprons	[42]
7	Prohibition of sale of antibiotics without prescription/judicious use of antibiotics	[69,74]
8	Improved surveillance system for antimicrobial resistance	[75]
9	Adherence to infection control best practices	[76]
10	Education of hospital staff and community for infection control/proper drug use and maintenance of hygiene/contact precaution	[77,78]

antimicrobial and biocidal agent[80]. Biofilm producing virulence is also found associated with aminoglycoside resistance genes. Rajamohan *et al*[25] demonstrated an increased biocide resistance and multidrug resistance in *A. baumannii* associated with the ability to form stronger biofilms. In part, the resistance may be increasing due to low penetration of antimicrobials into biofilms, in addition to acquisition of resistance genes through mobile genetic elements[81]. The continuous presence of high selection pressure of antimicrobials and disinfectants in intensive care units is also been correlated to increased multidrug resistance, strong biofilm abilities, and survival of these variant within such biofilms[16,82]. Thus control of such variants are a challenge, and difficult with routine antimicrobial and biocidal agents.

Microbiology laboratories can provide frontline surveillance for antibiotic resistance and are therefore useful in combating nosocomial infections[83]. Rapid, accurate analysis of antimicrobial susceptibility will be useful in determining the precise use of antimicrobial agents. Hence, clinical input from a microbiologist is necessary to keep one step ahead in controlling nosocomial infections. Periodic surveillance by molecular typing of isolates from patients is recommended for early detection of an epidemic strain, which consequently serves as an effective control measure[84]. Empiric antimicrobial therapy based on such observations is useful when laboratory findings are impeded for one reason or another[85,86]. Such therapy has been successful against pneumonias, ventilator-associated pneumonias, and bloodstream infections caused by *A. baumannii*, especially in critically ill patients[87-90], although some failures have also been reported, and caution is advised[91]. Empiric carbapenem therapy is a popular example of such a regime[14,92,93]. With the rise of carbapenem resistance in MDR phenotypes, this approach seemingly faces difficulties[14]. MDR is a common phenomenon associated with *A. baumannii* that is on the increase[10,94-96]. There are no clear guidelines to treat *A. baumannii* infections, and antipseudomonal broad-spectrum penicillins and cephalosporins and the members of other categories such as monobactams, aminoglycosides, fluoroquinolones, carbapenems, glycylcyclines, polymyxins, and β-lactamase inhibitors are used to control infections involving *A. baumannii*. Selection of the appropriate antimicrobial agent for empirical therapy is therefore challenging and has to be based on local institutional and hospital findings. Treatment decisions are usually made on a case-by-case basis by a health care provider. Empirical treatment therefore is likely to differ for a given geographic location[97]. Antibiotic susceptibility testing and other phenotypic tests for detecting double-disk synergy should be used as a guide, in addition to approved governing guidelines. Institutional data mining and retrospective analysis are often of great help in this regard and are advised by Towner[10].

Because of the limited choice of antimicrobial agents, *A. baumannii* infections are treated mainly with extended-spectrum β-lactams; β-lactams with β-lactamase inhibitors such as tazobactam or sulbactam; and carbapenems. Colistin and sulbactam are still relatively effective against infection caused by MDR *A. baumannii*, but an anticipatory fear of the development of resistance is increasing in ICUs. Peptides and other novel antibacterial agents are in the experimental phases. A combination therapy (dual or triple therapy) of a carbapenem with sulbactam, tobramycin, colistin, and aztreonam is being assessed in laboratory synergy studies, but clinical trials are required before one can adopt such combination regimens[98]. A study containing pharmacokinetic-pharmacodynamic profiling of four antimicrobial drugs against *A. baumannii* suggested that a combination involving carbapenem is required for effective therapy[99]. A glycopeptide (vancomycin or teicoplanin)-colistin combination was found to be highly active (synergism) against *A. baumannii* both *in vitro* and in a simple animal model[100]. A complicated case of persistent MDR *A. baumannii* central nervous system infection (ventriculitis) was resolved by a prolonged triple combination therapy involving intraventricular colistin and tobramycin plus intravenous colistin, rifampin, and vancomycin[101]. In murine pneumonia and rabbit meningitis models of *A. baumannii* infection, imipenem or sulbactam were found to be appropriate for combination therapy when used with rifampin[102]. A comparative *in vitro* study of synergistic activities also demonstrated that imipenem has better synergism with colistin than does amikacin or ampicillin/sulbactam against carbapenem-resistant *A. baumannii*[103]. In another study, tigecycline, a recently developed novel broad-spectrum antibacterial agent, was used (off-label indication) in combination therapy to treat MDR *A. baumannii* superinfection. However, the studies had several limitations such as retrospective design, small number of patients, and tigecycline as a part of the combination[56]. Despite its association with nephrotoxicity, colistin has been used by different modes of administration. Nebulized colistin was found to be more efficient in *A. baumannii* pulmonary infections when administered solely in nebulized

Table 2 Some of the commonly reported mechanisms of resistance in *Acinetobacter baumannii* from different geographic locations

Category of mechanism	Gene involved	Geo-location	Ref
ESBL	*PER-1* type	Hungary, India, Turkey, Korea, France, Belgium, Romania	[114,127-132]
ESBL	*VEB-1* type	Belgium, France	[131,133]
ESBL	*KPC* type		[134]
ESBL	*CTX-M-2* type	Japan	[135]
Carbapenemase	*OXA* type	United Kingdom, transcontinental	[120,136]
Carbapenemase	*OXA* type	United States,	[119,122,123,137]
Carbapenemase	*OXA-51* type	United Kingdom, France, Iraq, United States	[123,138-140]
Carbapenemase	*OXA-23* type	United Kingdom, China, United States	[141,142]
Carbapenemase	*OXA-40* type	Spain, United States	[123,143]
Carbapenemase	*OXA-58* type	Greece, Italy, Bolivia	[144,145]
Carbapenemase (multiple)	*OXA, IMP, VIM*	Korea	[146]
Carbapenemase, MBL	*NDM*	Israel, Germany	[147,148]
Carbapenemase, MBL	*VIM*	Poland	[149]
Carbapenemase, MBL	*IMP*	Japan, Brazil	[150,151]
Carbapenemase, MBL	*SIM*	China	[152]

ESBL: Extended-spectrum β-lactamase; MBL: Metallo-β-lactamase

form or in combination with intravenous colistin against intravenous colistin alone[104]. Colistin is still considered a good choice against MDR *A. baumannii* compared with ampicillin/sulbactam[105-107] or rifampin+imipenem[107]. The nephrotoxicity associated with colistin is reported to be reversible and less frequent than once thought. Neurotoxicity is rare, although more posological research is needed[33]. At present, no new drugs that could be available in 5 years are currently in the pipeline; therefore, combination regimens of antibiotics are the only resources to combat this infection.

ANTIMICROBIAL RESISTANCE IN *A. BAUMANNII*

The three major forces that drive antimicrobial drug resistance are failure to maintain hospital hygiene, selective pressure due to irrational use of antibiotics, and mobile genetic elements encoding the bacterial resistance mechanism[96]. The resistance among *A. baumannii* strains to β-lactam agents is of great concern among clinicians. The β-lactams are broadly accepted for treatment because of the availability of a wide range of drugs, their broad spectrum of activity, minimum side effects, and most importantly, their relatively low cost in developing countries of Africa, Asia and Latin America. The restriction on the use of these agents because of the emergence of resistance is a loss to the community and a great blow to the health care system. The mechanism of resistance to β-lactam in *A. baumannii* can be attributed to an intrinsic property or an acquired phenomenon. This organism is a known reservoir of multiple plasmids carrying antibiotic resistance markers[16,95]. The later mobile genetic element is of concern because the acquisition of resistance genes can radically change the scenario of drug resistance. *Acinetobacter* spp are also known to donate resistance-plasmids and are therefore likely to rapidly disseminate resistance among other commensals or pathogens.

Acinetobacter harbors multiple mechanisms of drug resistance. The mechanism of resistance to β-lactam agents in *A. baumannii* involves production of a variety of chromosomal or plasmid-mediated β-lactamases, especially extended-spectrum β-lactamase (ESBL), alteration of drug-binding proteins, permeability changes in the cell membrane, loss of porins, and efflux pump, of which the presence of an array of β-lactamases is the predominant weapon[108-111]. *Acinetobacter* produce a variety of β-lactamases. The main mechanisms of resistance to extended-spectrum cephalosporins in *A. baumannii* are the over-expression of chromosomal cephalosporinases and plasmid-encoded Ambler class A, B, and D β-lactamases[112]. ESBL-producing *A. baumannii* strains are now reported from various geographic areas of the world. These include the TEM type, SHV type, CTX-M type, PER-1, and VEB-1 β-lactamases. The prevalence of ESBLs is much higher in the isolates from ICUs than in isolates from other hospital sites[113,114]. *A. baumannii* produces a variety of extended-spectrum β-lactamases, depending on its geographical location. The PER-1 ESBLs were from Turkey, Korea, Russia, Romania, Belgium, France, and India; VEB-1, from France and Belgium; TEM-116 and TEM-92, from China and Italy, respectively; SHV-12 from the Netherlands; CTX-M-2 and CTX-M-43 from Korea and Bolivia (Italy), respectively[114-118]. Table 2 demonstrates in brief the representative mechanisms reported from different geographic locations. It was believed that ESBL-producing *A. baumannii* strains remain susceptible to carbapenems. However, OXA-type ESBL-producing *A. baumannii* isolates resistant to carbapenems have been widely reported, including from the United States[119-122], that carry insertion sequence, IS*Aba1* upstream to OXA-like genes[123]. Although resistance in *A. baumannii* to polymyxins such as colistin is rare, recent reports suggest that an underlying mechanism of moderate resistance to colistin involves point mutation in pmrB, upregulation of pm-

rAB, and expression of pmrC, which lead to phosphoethanolamine modification of lipid A[33,124]. This finding means that we will not be able to use many more β-lactam drugs, which will further limit our options. Among carbapenem-resistant MDR *A. baumannii*, colistin is often the last resort. Recent findings suggest a slow rise of colistin-resistant isolates lead to Pan-drug resistant organisms[125]. With the help of rapid and powerful tools such as high throughput sequencing technologies *e.g.*, whole-genome sequencing, one can elucidate the origin of large outbreaks of such resistant pathogens, and the exact genetics behind resistance mechanisms[125,126].

FUTURE PROBLEMS

A contentment of multidrug resistance and their dissemination in *Acinetobacter baummannii* is not an easy task. While multiple drug resistance is increasing in this pathogen, and carbapenem resistance is rapidly spreading cross-continentally, there is a sharp decline in development of new antimicrobial agents that can control MDR *A. baumannii*. There is no new drug in pharmaceutical pipeline or none of the FDA-approved antimicrobial compounds tested had appreciable effect in control of MDR *A. baumannii*. The existing antimicrobials also failed to control the resistance development and effective elimination of MDR variants. A rational synergistic approach of some of the combination therapies although working, needs more in-depth understanding, and systematic studies are required in order to control probably outbreaks. Creation of pan-drug resistant variants will have to be avoided, and efforts on new anti-acinetobacter drug development would be invested.

CONCLUSION

Microbiological surveillance facilitates the ability to monitor changes in the trends of dominant microorganisms and their antimicrobial susceptibilities in hospitals. It helps to detect recent resistance mechanisms in these pathogens and to formulate antimicrobial usage policies for the hospital and adds to the epidemiological information about these organisms in particular regions of the country.

The MDR *Acinetobacter* clinical isolates, especially in the ICUs of hospitals, are a serious public health concern worldwide, and responsible for high mortality. The geographic variation in resistance patterns emphasizes the importance of local surveillance in determining the most suitable therapeutic option to treat *Acinetobacter* infections. The lack of therapeutic options for treating MDR organisms calls for systematic pharmacokinetic and pharmacodynamic studies of rational combination therapies until new, powerful drug appear in clinical practice for this purpose.

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REVIEW

Physiological functions and clinical implications of fibrinogen-like 2: A review

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Abstract

Fibrinogen-like 2 (FGL2) encompasses a transmembrane (mFGL2) and a soluble (sFGL2) form with differential tertiary structure and biological activities. Typically, mFGL2 functions as prothrombinase that is capable of initiating coagulation in tissue without activation of the blood clotting cascade, whereas sFGL2 largely acts as an immunosuppressor that can repress proliferation of alloreactive T lymphocytes and maturation of bone marrow dendritic cells. Protein sequences of FGL2 exhibit evolutionary conservation across wide variety of species, especially at the carboxyl terminus that contains fibrinogen related domain (FRED). The FRED of FGL2 confers specificity and complexity in the action of FGL2, including receptor recognition, calcium affiliation, and substrate binding. Constitutive expression of FGL2 during embryogenesis and in mature tissues suggests FGL2 might be physiologically important. However, excessive induction of FGL2 under certain medical conditions (*e.g.*, pathogen invasion) could trigger complement activation, inflammatory response, cellular apoptosis, and immune dysfunctions. On the other hand, complete absence of FGL2 is also detrimental as lack of FGL2 can cause autoimmune glomerulonephritis and acute cellular rejection of xenografts. All these roles involve mFGL2, sFGL2, or their combination. Although it is not clear how mFGL2 is cleaved off its host cells and secreted into the blood, circulating sFGL2 has been found correlated with disease severity and viral loading among patients with human hepatitis B virus or hepatitis C virus infection. Further studies are warranted to understand how FGL2 expression is regulated under physiological and pathological conditions. Even more interesting is to determine whether mFGL2 can fulfill an immunoregulatory role through its FRED at carboxyl end of the molecule and, and vice versa, whether sFGL2 is procoagulant upon binding to a target cell. Knowledge in this area should shed light on development of sFGL2 as an alternative immunosuppressive agent for organ transplantation or as a biomarker for predicting disease progression, monitoring therapeutic effects, and targeting FGL2 for repression in ameliorating fulminant viral hepatitis.

Key words: Fibrinogen-like 2; Prothrombinase; Immunosuppressor; Infectious disease

Core tip: Fibrinogen-like 2 (FGL2) protein promotes coagulation as a prothrombinase, or acts as an immunosuppressor to repress function of T lymphocytes and dendritic cells and induce apoptosis of B lymphocytes. Ectopic expression of FGL2 has been proven relevant for the pathogenesis of viral infections. Induction of FGL2 in response to pathogen invasion causes focal prothrombin activation and fibrin deposition. This process may lead to inflammation, microvascular thrombosis, and subsequent organ failure. FGL2-mediated immunosuppression can facilitate pathogen proliferation and expansion. The understanding of FGL2-mediated

pathophysiology offers an insight into biomarker development and clinical intervention of FGL2-associated medical conditions such as viral hepatitis.

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INTRODUCTION

Fibrinogen-like 2 (FGL2), also known as fibroleukin, is a multifunctional protein. FGL2 has been found to be not only physiologically important[1-3], but also involved in pathogenesis of viral infections[4,5], pregnancy failure[6], autoimmune disorders[7,8], allograft rejections[9], and tumor growth[10]. Alternations in FGL2 expression or structure are tied to several highly virulent viral infections, including human immunodeficiency virus (HIV) infection, severe acute respiratory syndrome (SARS), and hepatitis B and C[4,5,11]. In this review, constitutive expression and physiological roles of FGL2 that have been identified to date will be illustrated to help understand pathological properties of FGL2 during pathogen invasion when ectopic expression of FGL2 occurs. While FGL2 might have a potential to be used as a biomarker or therapeutic target, some research gaps will be explored to expand possible clinical applications of FGL2. Although signal transduction pathways involved in regulation of *FGL2* transcription and post-transcriptional modifications are important, in-depth discussion of these molecular mechanisms is not in the scope of this review.

STRUCTURE AND FUNCTION

Several studies have suggested that FGL2 is highly conserved, sharing over 70% homology among human, mouse and rat[12-15]. A phylogenetic tree analysis suggests an even closer evolutional relationship between human *Fgl2* and pig *Fgl2*[16]. This extraordinary evolutionary conservation across different species suggests that FGL2 might be an indispensable protein with critical biological function(s).

Two distinct forms of the FGL2 protein have been identified, membrane-associated FGL2 (mFGL2) and soluble FGL2 (sFGL2). mFGL2 integrates with phospholipids of cellular membranes and is expressed as a type Ⅱ transmembrane protein[14], while sFGL2 can be secreted into the vasculature. Native sFGL2 exists as an oligomer consisting of four disulfide-linked FGL2 monomers[1,17]. The difference in the tertiary structure between mFGL2 and sFGL2 suggests that the two forms of FGL2 may function differently.

mFGL2 has been found to be expressed on endothelial cells, epithelial cells, macrophages, and dendritic cells[18-20]. The first 19 to 26 amino acids at the N-terminus are highly hydrophobic and predicted to serve as the transmembrane domain of mFGL2[14,21]. Three tentative serine protease active sites at positions 91, 142, and 423 are conserved between human and mouse FGL2[14]. The residue serine 91 of human FGL2, corresponding to the serine 89 of murine FGL2, has been revealed to be capable of cleaving prothrombin into thrombin[14,22]. FGL2 was thus speculated and has been demonstrated to function as a prothrombinase[15,22,23]. FGL2 activates prothrombin to generate thrombin that in turn converts fibrinogen into fibrin, a process equivalent to factor Ⅱ (F Ⅱ) activation[15,22,23]. However, unlike F Ⅱ, the proteolytic activity of FGL2 is independent of factor *X* and cannot be inhibited by antithrombin Ⅲ[22,24]. Instead, full proteolytic activity of FGL2 is contingent on its physical association with membrane phospholipids, factor Va, and calcium[22]. Therefore, prothrombinase activity appears to be intrinsic to mFGL2, but similar function of sFGL2, if there is any, has yet to be demonstrated.

sFGL2 is known to be secreted by cytotoxic and regulatory T lymphocytes upon activation, but not by helper T lymphocytes and B lymphocytes[8,12,17,19,21,25]. A stretch of hydrophobic amino acids at N-terminus of FGL2 may serve as signal peptide for sFGL2 secretion[14], but the mechanism whereby sFGL2 is cleaved and released outside of the host cell remains to be determined. Glycosylation of the amino acids at positions of 172, 228, 256, and 329 were found to be critical to maintain the solubility of sFGL2[16]. Previous studies indicate that sFGL2 lacks procoagulant activity of mFGL2[12,17], rather, it functions largely as an immunosuppressor or pro-apoptotic effector molecule[26]. sFGL2 has been shown to inhibit maturation of bone marrow-derived dendritic cells; maintain immunosuppressive activity of regulator T cells (Tregs); and suppress T cell proliferation in response to the stimulation by alloantigens, anti-CD3/Cd28 antibodies, or CoA[8,26,27]. A fibrinogen related domain (FRED) at the carboxyl terminus is believed to account for the immunosuppressive activity of sFGL2 as a monoclonal antibody against FRED abrogated the sFGL2-mediated suppression of T cell proliferation[26]. sFGL2 binds specifically to Fc gamma receptor (FcγR) Ⅱ B/CD32. This receptor is expressed on sinusoidal endothelial cells (SECs) within the liver[28]; glomerular mesangial cells within the kidney[29]; and immunoregulatory cells such as dendritic cells (DCs), B lymphocytes, macrophages, and activated T lymphocytes[16,30,31]. Binding of sFGL2 to SECs, glomerular mesangial cells, B lymphocytes, or macrophages caused apoptosis of the target cells[8,28,30]. Binding of sFGL2 to bone marrow DCs can inhibit lipase (LPS)-induced DC maturation[30] and thereby impairs the ability of DCs to stimulate alloreactive T cell proliferation[26]. In addition, sFGL2 can bind directly to T lymphocytes to inhibit their proliferation and polarize allogeneic immune response toward a Th2 cytokine profile by inducing interleukin 4 (IL-4)/IL-10 while inhibiting IL-2/interferon gamma (IFN-γ) produc-

tion[8,26]. Consistent with these observations, the levels of Th2 cytokines and the activity of DCs, B lymphocytes, and T lymphocytes have all been found to be increased in FGL2-deficient mice[8].

PHYSIOLOGICAL ROLES

Dissecting potential functions of a protein under normal physiological conditions sets a foundation for understanding of pathological properties of this molecule. Constitutive expression of FGL2 has been detected in the heart, lung, small bowel, spleen, ovary, uterus, liver, and kidney[13,32]. Expression of FGL2 is regulated tightly and associated with several physiological processes, including sperm maturation[1], embryo development[3,33], and smooth muscle contraction[2,34].

FGL2 might play a protective role during sperm maturation in epididymis[1]. The expression of *Fgl2* messenger RNA (mRNA) under normal physiological conditions has been identified in the tubule principal cells of hamster epididymis[1]. FGL2 was found to be secreted from the principal cells into the tubule lumen where sFGL2 binds specifically to the nonviable, but not the viable, spermatozoa[1]. This process forms sFGL2-protein complex that coats and envelops dying sperms to restrict release and spread of detrimental enzymes and immunogenic molecules from defective spermatozoa. Nevertheless, FGL2-deficient male mice were still fertile[35], suggesting that the potential protective role of FGL2 is limited, or becomes prominent only under certain medical conditions when increased apoptosis of spermatozoa occurs.

Expression of FGL2 has been shown to change dynamically during murine embryogenesis[3]. FGL2 was first detectable at the implantation site at E5.5 of gestation among CBA mice that exhibited the rate of pregnancy failure equivalent to that expected on the basis of embryonic chromosome abnormalities[3]. By E6.5, the embryo itself became positive for FGL2, but the level diminished at the maternal-fetal trophoblastic interface by E7.5 and was barely detectable in the developing embryo at E8.5 to E9.5[3]. By E13.5, however, FGL2 was detected in somites (future vertebra) and adjacent neural tube within the embryo[3]. This spatio-temporally coordinated expression of FGL2 , in forms of mFGL2 or sFGL2, during embryonic development suggests that FGL2 might play a physiological role in embryogenesis. Congruent with this speculation, the level of *FGL2* messenger RNA was higher in gravid myometrium tissues from pregnant women than in the hysterectomy samples from premenopausal non-pregnant women ($P < 0.001$)[36]. The physiological function of FGL2 has been further demonstrated in animal studies. Knocking-out *Fgl2* gene (*Fgl2*$^{-/-}$) in mice led to higher rate of pregnancy failure than wild type (*Fgl2*$^{+/+}$) mice[33]. Early miscarriage of mouse embryos between the time of implantation (E4.5) and formation of vascularized placenta (E9.5) were associated with absence of physiological expression of FGL2[3], suggesting that FGL2 might aid embryo implantation and placenta development.

Given that sFGL2 can suppress T cell activation[26], it is likely that pregnancy failures among *Fgl2*$^{-/-}$ mice might have been caused by loss of local suppression of classical T cells or natural killer T cells that otherwise might contribute to immune rejection of developing fetus half of whose antigens are encoded by paternal genes[37]. In addition, mFGL2 might function as prothombinase to improve coagulation and reduce hemorrhage at the implantation site that is often seen in FGL2-deficient embryos but not in wild type embryos[3,23,38].

FGL2 might be involved in modulation of vascular and nonvascular smooth muscle contraction. Expression of FGL2 was detected in mouse cardiomyocytes[2]. *Fgl2*$^{-/-}$ murine embryos had significantly lower heart rates than *Fgl2*$^{+/+}$ embryos[2]. About 33% of *Fgl2*$^{-/-}$ pups died within 3 d after birth due to acute congestive cardiac failure resulted from myocardial contractile dysfunction[2]. These data suggest that FGL2 is critical for normal myocardial function during prenatal and postnatal development in mice[2], but it is not clear how FGL2 deficiency is linked to abnormal myocardial contraction. O' Brien *et al*[34] investigated expression of FGL2 in biopsies of human uterine myometrium incised during cesarean delivery [pregnant not in labor (PNL)] or at intrapartum [pregnant in labor (PL)]. They noticed that both *FGL2* mRNA and FGL2 protein were expressed more prominently in PL samples than in PNL samples. Interestingly, up-regulation of thrombin receptors, F2R and F2RL3, were found to be correlated with FGL2 elevation in the myometrium in labor[34]. Increased level of FGL2 can lead to thrombin accumulation in myometrium. Thrombin in turn binds to these receptors and causes cytosolic enrichment of calcium[39]. This process may ultimately result in myometrial smooth muscle contraction[39,40]. Pretreatment with a thrombin-specific inhibitor hirudin prevented myometrial contraction[40]. Therefore, FGL2 appears to modulate vascular and nonvascular muscle contraction through generation of thrombin.

PATHOGENESIS

Disturbances in the tight control that balances the time and location of constitutive FGL2 expression have been implicated in the pathogenesis of pathogen invasion[4,5,11], miscarriage/pre-eclampsia[6,41,42], allograft rejections[9], autoimmune diseases[7,8], and tumor growth[10,43]. Pathogenesis of these disorders share some common features rooted from ectopic expression of mFGL2 or sFGL2. This section focuses on potential mechanisms behind the pathogenesis of the infectious diseases that involve abnormal activities of mFGL2, sFGL2, or both.

HIV-1 infection typically advances through acute phase to asymptomatic stages and finally to full-blown acquired immune deficiency syndrome (AIDS). Acute stage is characterized by elevated expression of genes involved in immune activation and defenses, resulting

in partial control of HIV infection and progression to asymptomatic stage. Expression of a host of immunosuppressive genes including *FGL2* is activated at the asymptomatic stage[5]. Up-regulation of sFGL2 may help to dampen the immunopathological consequences of sustained immune activation during acute phase of HIV infection, however, the host immune system is probably too naïve to assume that their mission is over and wind down immunosurveillance by turning on the expression of *FGL2* and other immunosuppressive genes. Elevation of FGL2 at the asymptomatic stage might have facilitated HIV to escape the host immune protection and thus leading to uncontrolled viral proliferation and AIDS[5].

The SARS coronavirus (SARS-CoV) is the etiologic agent responsible for the outbreak of SARS in Asia in 2003. The infection resulted in a mortality rate of 50% among patients over 60 years of age[44]. A homozygous mutation (dbSNP ID: rs2075761; JSNP ID: IMSJST003521) at the amino acid position 53 (FGL2 G53E) appears to be weakly associated with level of nasopharyngeal shedding of SARS-CoV (P = 0.041)[45]. Subsequent studies from the same group confirmed that FGL2 G53E was a dominant risk variant for SARS-CoV infection[46]. Individuals carrying this mutation had about 40% higher SARS-infection rate than those without this mutation (P < 0.0001)[46].

It is worth noting that the G53E point mutation is not located near known important functional motifs, including FRED, glycosylation sites, and serine prothrombinase sites. There is no report to date exploring whether and how the G53E mutation might affect the function of sFGL2. Nevertheless, SARS-CoV was shown to be capable of activating transcription of *FGL2* gene[47]. Transfection of plasmids expressing the nucleocapsid protein of SARS-CoV into human macrophage cell line or African green money kidney epithelial cells activated *FGL2* expression[47]. However, Siu *et al*[48] was not able to reproduce the results in the Vero cell line, nor in human embryonic kidney cells or cultured human airway epithelial cells. Therefore, it remains controversial as to whether SARS-CoV regulates *FGL2* transcription. Both SARS-CoV and murine hepatitis virus strain 1 (MHV1) are categorized as group 2 coronaviruses[49] and produced very similar pathological features. MHV1 infection of mice is thus proposed as an animal model for SARS research[50]. A study *in vivo* in A/J mice suggested that FGL2 might contribute to the pathogenesis of SARS-like severe pulmonary disease induced by MHV1[50]. However, there has been no direct evidence indicating that FGL2 might be involved in SARS infection.

FGL2 may contribute to pathogenesis of human hepatitis B virus (HBV) and hepatitis C virus (HCV) infections. Patients with HBV or HCV infection have higher levels of FGL2 than healthy controls[4,11,51]. Strong fibrin deposition and necrosis were co-localized with robust FGL2 expression in liver biopsies from 21 out of 23 patients with acute-on-chronic hepatitis B characterized by recurrent flares of hepatocellular injury, but not among all 13 patients with minimal chronic hepatitis that exhibited no major active liver pathology[23,51]. Similarly, the plasma levels of FGL2 were over 2-fold higher among patients with HCV infection (n = 80) than healthy controls (n = 30, P < 0.001)[4].

The expression of FGL2 seemed to correlate with the progression of viral hepatitis. Mean levels of plasma sFGL2 were nearly 3-fold higher among HCV-patients with cirrhosis (n = 60) than those without cirrhosis (n = 20, P = 0.001)[4]. Fibrosis stage is an established indication for disease severity and efficacy of anti-viral treatment. Plasma levels of sFGL2 among HCV-patients with advanced fibrosis (stage 3-4, n = 22) were twice that of patients with moderate fibrosis (stage 2, n = 19, P = 0.01) and over 3-fold higher than those with mild fibrosis (stage < 1, n = 35, P = 0.001)[4]. However, the levels of FGL2 among HCV-patients with inactive alcoholic cirrhosis were comparable to the controls[4], suggesting that it is the activity and progression of HCV infection, not the end stage cirrhosis,that accounts for the high levels of FGL2 among HCV-patients.

How does HBV-infection or HCV-infection lead to increased FGL2 expression, and why do high levels of FGL2 correlate with the disease progression? Han *et al*[11] found that HBV core protein or *X* protein were both capable of binding directly to the promoter of *FGL2* gene and activating its transcription in a hepatocellular carcinoma cell line. On the other hand, sFGL2 has been found to function as an immunosuppressor to inhibit maturation of dendritic cells, reduce production of IFNγ, and impair proliferation of alloreactive T lymphocytes among the patients with HBV or HCV[52-54]. In contrast, FGL2-deficiency was associated with the development of T cell leukemia/lymphoma[55]. Collectively, HBV/HCV might up-regulate sFGL2 expression. High levels of sFGL2 in turn might jeopardize the host immune integrity and thus may facilitate viral replication and expansion. In addition, FGL2-mediated accumulation of fibrin could restrain or block blood flow in the liver and cause hepatocyte necrosis or even liver failure[23,51].

Observations from animal studies have provided further insights into the pathogenesis of viral hepatitis. Mouse hepatitis virus 3 (MHV3)-a member of Coronaviridae-has served as a model for dissecting pathological determinants of diseases caused by coronaviruses. *Fgl2* mRNA was detected in Kupffer (macrophage) cells and reticuloendothelial cells in the liver of BALB/cJ mice within 8 h post MHV3-infection[56]. FGL2 protein was detected within 24 h following MHV3 infection in endothelium of intrahepatic veins and hepatic sinusoids where concomitant fibrin deposition and subsequent focal hepatocyte necrosis occurred[56]. Ning *et al*[57,58] demonstrated that the nucleocapsid protein of MHV3 was capable of activating transcription of mouse *Fgl2* gene *in vitro*. Serum level of sFGL2 was shown to correlate with liver cytopathology among the mice infected by MHV3[19]. More compelling evidence suggesting that

FGL2 might contribute to pathogenesis of viral infection comes from the elegant studies by Shalev *et al*[27] and by Marsden *et al*[23], who have demonstrated that MHV3 caused fibrin deposition and hepatocellular necrosis only in *Fgl2*$^{+/+}$ mice but not in *Fgl2*$^{-/-}$ mice. Further, they have showed that administration of anti-FGL2 monoclonal antibody (mAb) improved liver histology and survival rate[27]. Similarly, target deletion of *Fgl2* gene in C57BI/6 mice[23] or depletion of *Fgl2* mRNA by introducing dual short hairpin RNA into BALB/cJ mice before exposing to MHV3 can alleviate liver pathogenesis and improve survival rate[59].

Given FGL2-mAb directed to the FRED region that conveys immunosuppressive activity reduced MHV3 viral titers among infected mice[27], it is reasonable to postulate that FGL2-mediated-immunosuppression might play a role in viral hepatitis. However, several lines of evidence suggest that FGL2-mediated immunosuppression is not a major determinant in the pathogenesis of viral hepatitis. First, MHV-3 viral loads in the livers did not vary between *Fgl2*$^{+/+}$ and *Fgl2*$^{-/-}$ mice[23]. Second, although administration of FGL2 neutralizing antibodies abrogated hepatitis in mice infected by MHV3, a high viral load persisted[60]. Finally, in spite of similar MHV3 viral load between the spleen and the liver of MHV3-infected BALB/cJ mice, the pathogenesis was restricted in the liver with complete absence of disease in the spleen. Recent studies have demonstrated that the progression of fulminant viral hepatitis usually exhibits a similar pattern, viral-induced up-regulation of the *Fgl2* gene precedes focal deposits of fibrin in sinusoids, followed by accumulation of inflammatory cells and focal hepatocyte necrosis. The roles of FGL2 in the rapid development of confluent multicellular hepatic necrosis are probably fulfilled through several interrelated processes: (1) FGL2-mediated fibrin deposition may hamper or terminate sinusoidal blood flow and cause hepatocyte necrosis[61]; (2) FGL2 may bind directly to the (FcγR) IIB receptors on sinusoidal endothelial cells and trigger cellular apoptosis[28]; and (3) FGL2 may stimulate inflammation through generation of thrombin. Thrombin is known to be able to stimulate endothelial cells to produce IL-8[62,63]. Knocking-down *Fgl2* using *Fgl2* RNA interference (RNAi) caused a reduction of LPS-mediated IL-8 production[20]. IL-8 is a potent chemo-attractant for polymorphonuclear leukocytes that have been identified at the sites of FGL2-mediated inflammation.

It is puzzling why FGL2 induction and hepatocellular necrosis occurred only among BALB/cJ mice but not among A/J mice infected by MHV3[23,51]. These BALB/c-susceptible and A/J-resistant phenotypes might be attributable to several factors. First, MHV3 induced significantly greater apoptosis of macrophages from A/J mice than from BALB/cJ mice[64]. MHV3 is propagated in macrophages[19]. Apoptosis of macrophages might decrease the extent of viral replication and result in higher MHV3 viral load in BALB/cJ than in A/J mice. Additionally, macrophages are a source of FGL2 production[19,56,64]. *Fgl2*$^{+/+}$- macrophages exhibited a robust procoagulant response to MHV3; whereas procoagulant activity from *Fgl2*$^{-/-}$ macrophages exposed to MHV3 was comparable to the control levels[23]. Therefore, reduction in the number of macrophages due to apoptosis might translate into a diminished level of FGL2. In accordance with this hypothesis, apoptosis of macrophages mediated by target ablation of the gene encoding an inhibitory receptor B and T lymphocyte attenuator (*BTLA*$^{-/-}$) repressed FGL2 induction by MHV3 in the liver[19]. The reduction of FGL2 was associated with decreased tissue lesions and mortality among *BTLA*$^{-/-}$ mice infected with MHV3[19]. Adoptive transfer of macrophages into *BTLA*$^{-/-}$ mice increased their mortality rates close to those seen in *BTLA*$^{+/+}$ mice infected by MHV3[19]. This finding appears to be clinically relevant. Biopsies analysis indicates that the numbers of CD68^{+} macrophages were strikingly higher in the liver from patients with active HBV-infection than from normal controls and the patients with inactive chronic hepatitis[65]; Next, MHV3 induced over 100-fold lower level of *Fgl2* mRNA and significantly less amount of FGL2 protein in macrophages isolated from A/J mouse than from BALB/c mice (Fung *et al* 1991)[66]; Thirdly, baseline number and percentage of CD4^{+}Foxp3^{+}-Tregs in the spleen and thymus were 1-fold to 2-fold greater among BALB/cJ mice than among A/J mice[27]. These Tregs expressed FGL2 and depended on FGL2 for their immunosuppressive activity[8,27]; therefore, higher numbers of Tregs in BALB/cJ mice may confer faster viral replication and worse pathology. Adoptive transfer of *Fgl2*$^{+/+}$ Tregs or *Fgl2*$^{+/+}$ splenocytes that contain Tregs into *Fgl2*$^{-/-}$ mice 1 h before exposing to MHV3 recapitulated the susceptible phenotype seen in *Fgl2*$^{+/+}$ mice infected by MHV3[27]; Finally, sFGL2 binds to the inhibitory FcrRIIB receptor on DCs and B cells from BALB/cJ mice but not the DCs and B cells from A/J mice due to an allelic polymorphism of the FcγRIIB receptor in A/J mice[31]. As a result, FGL2-mediated immunosuppression might be less significant among A/J mice than among BALB/c mice, which may explain why A/J mouse is able to clear MHV3 shortly after the viral infection[67].

Interestingly, although A/J mice can clear MHV3 by 10 d to 14 d post infection, pretreatment of A/J mice with corticosteroids, methylprednisolone, abolished their resistance to MHV3 and the animals died within 10 d of infection[67]. It turned out that methylprednisolone stabilized *Fgl2* mRNA and hence increased accumulation of *Fgl2* mRNA, which in turn translates into more FGL2 protein[67,68]. Immunofluorescence analysis of the liver tissue from methylprednisolone-treated and MHV3-infected A/J mouse showed increased expression of FGL2 in areas of inflammation around hepatic sinusoids[67].

Taken together, the level of FGL2 correlates positively with the development and severity of typical MHV cytopathology[51,64]. Animal studies have suggested that elevation of FGL2 might be one of critical determinants of susceptibility to hepatitis virus infection[51].

POTENTIAL VALUE AS A BIOMARKER OR THERAPEUTIC TARGET

It would be highly desirable to measure a substance in readily available specimens such as blood or urine that would lead to disease diagnosis, reflect disease burden, correlate with therapeutic results, or simply be utilized as a surveillance marker to predict disease prognosis. FGL2 appears to be such a candidate. Variations in the plasma level of FGL2 among healthy human volunteers were minimal, regardless of race, gender, or age[4]. In contrast, plasma levels of FGL2 correlated positively with HCV titers and degree of inflammation in the liver[4]. The level of FGL2 dropped significantly following an effective anti-viral therapy among patients with biopsy-proven HCV hepatitis (n = 32, $P < 0.001$)[4]. Furthermore, as discussed previously, FGL2 expression has been found to be associated with progression and severity of disease. HCV-patients with cirrhosis had significantly higher levels of FGL2 (164.1 ± 121.8 ng/mL, n = 60) compared with patients without cirrhosis (57.7 ± 52.8 ng/mL, n = 20, P = 0.001) and patients with inactive end stage alcoholic cirrhosis (18.8 ± 17.4 ng/mL, n = 24, $P < 0.001$)[4]. Similarly, FGL2 was detected in peripheral blood mononuclear cells (PBMC) from 28 of 30 patients (93%) with severe hepatitis B, but only 1 of 10 (10%) patients with mild chronic hepatitis B[51]. FGL2-procoagulant activity was more than 10-fold higher on PBMCs from patients with acute-on-chronic hepatitis B than from healthy controls[51].

Plasma levels of FGL2 have been found to correlate with diseases other than viral hepatitis. For example, the levels of plasma FGL2 were significantly higher among patients with fatty liver disease than healthy controls[69]. Likewise, although the elevation of FGL2 was not associated with clinical features of systemic sclerosis, the mean serum level of FGL2 among patients with systemic sclerosis (28.7 ± 17 ng/mL) was significantly higher than that among healthy controls (11.4 ± 5.5 ng/mL, $P < 0.001$)[70].

Recent research has provided exciting insight into clinical application of FGL2 as a therapeutic target. Animal studies suggested that effective disease intervention could be achieved through modulation of FGL2 expression at DNA or protein level. For example, tail-vein injection of antisense plasmid complementary to the exon 1 of mouse *Fgl2* gene into BALB/cJ mice caused marked reduction of inflammatory cell infiltration, fibrin deposition, and hepatocyte necrosis[71]. All 18 mice receiving *Fgl2* antisense plasmid were alive on 3 d post MHV3-infection[71]. Six of 18 mice (33%) recovered from fulminant viral hepatitis[71]. In contrast, no mice in the control group (n = 18) survived beyond 3 d postinfection[71]. Similar effects have been observed by targeting FGL2 protein directly. Administration of FGL2-mAb resulted in a dose-dependent reduction of MHV3 viral titers among infected mice and improved liver histology and survival rate[27, 60].

Infections after organ transplantation remain a significant cause of mortality among the recipients[72,73]. For example, Sanders-Pinheiro *et al*[73] reported nearly 80% of kidney transplant recipients (n = 80) had infections and 53.8% of death resulted from infections. High rate of severe infections have also been seem among liver recipients with HIV/HCV-coinfection[74]. Current steroid or steroid-free immunosuppression scheme following an organ transplantation has been found to be associated with cardiovascular disease and infections[72,73,75]. Therefore, novel regimen is in great need to overcome or minimize adverse effects of immunosuppression[30]. Intravenous injection of recombinant sFGL2 into donor mice receiving skin transplantation prolonged the survival of skin allografts from 7.8 ± 1.99 d to 15 ± 2.56 d ($P < 0.001$)[30]. This finding might be clinically significant in that FGL2 could induce immune tolerance without relying on prolonged immunosuppression and thus help to reduce the risk of development of cardiovascular disease, infections, or cancer. Interestingly, monomeric FGL2 has been found to exhibit greater immunosuppressive activity than native oligomer sFGL2[16]. Monomeric FGL2 could be a better candidate in clinical usage than native sFGL2 in terms of its stronger potency, higher permeability and usually less antigenicity due to its lower molecular weight.

In summary, blood FGL2 might not be suitable for diagnosis as a disease-specific biomarker, but could emerge as an indicator to monitor disease progression and therapeutic effects for certain disorders such as HBV or HCV infection. While target-specific repression of mFGL2 expression has showed promising clinical implications for hepatitis therapy, sFGL2 may be used as novel immunosuppression agent for organ and tissue transplantations.

GAPS AND FUTURE DIRECTIONS

Although several reports have dealt with transcriptional regulation of *Fgl2* expression[11,57,76,77], it remains to be determined how the transcription and translation of *Fgl2* gene are regulated differentially in response to MHV3 infection across different cell types, tissues, and strains of animals. For example, Ding *et al*[56,61] reported that MHV3 induced *Fgl2* mRNA expression in the lung, liver, and spleen, but barely in the brain or kidneys of BALB/cJ mice despite of comparable viral titers in all tissues. At any time during the course of MHV-3 infection, FGL2 protein was only detectable in the liver but not any other tissues that were also positive for *Fgl2* mRNA[56]. In addition, MHV3 regulated FGL2 expression differentially even in the same type of cells from different strains of mice[78]. Exploring the mechanism responsible for cell- and tissue-specific expression of FGL2 may provide some insights into targeting FGL2 for more practical and effective clinical applications.

Further studies are needed to clarify unique and com-

mon functions between mFGL2 and sFGL2. Given that the FRED is conserved between mFGL2 and sFGL2, it is plausible to speculate that mFGL2 could also exhibit FRED-mediated immunosuppressive activity[26]. Although monomeric FGL2 has been found to be capable of suppressing allogeneic T cell proliferation[16], there is no report to date demonstrating that mFGL2 can act like sFGL2 to elicit immunologic response. Conversely, a valid question to ask is whether sFGL2 secreted by Tregs maintains prothrombinase activity, given FRED contains several prothrombinase-related functional motifs, including calcium ion loop and fibrinogen knob binding pocket[16]. FGL2 produced by the principle cells along epididymis epithelium can be secreted into the lumen where sFGL2 sequesters defective spermatozoa through forming polymerized protein matrix around dying cells[1]. However, it is not clear whether epididymal sFGL2 exhibits mFGL2-like prothrombinase activity and how the sFGL2 binds specifically to compromised spermatozoa to eliminate defective cells[1].

Sepsis is a life-threatening immune response to infection. Systemic coagulation and inflammation are hallmarks of this complication. Increased fibrin formation is also a characteristic clinical feature of sepsis[79,80]. Infection is often in concert with elevated production of inflammatory cytokines such as TNF-α and IFN-γ. TNF-α and IFN-γ have been found to induce *Fgl2* expression[77,81]. As discussed previously, FGL2 can activate coagulation and provoke inflammation through thrombin- and fibrin-generation, but the role of FGL2 in sepsis onset and progression needs further investigation.

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REVIEW

Microbial translocation, residual viremia and immune senescence in the pathogenesis of HIV-1 infection

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Abstract

The pathophysiological mechanisms that underlie the progression of human immunodeficiency virus-1 (HIV-1) disease to full-blown AIDS are not well understood. Findings suggest that, during HIV-1 infection, plasma lipopolysaccharide (LPS) levels, which are used as an indicator of microbial translocation (MT), are elevated throughout the acute and chronic phases of HIV-1 disease. The translocation of bacterial products through the damaged gastrointestinal barrier into the systemic circulation has been described as a driver of immune activation. In contrast, comorbidities that are associated with HIV-1 infection have been attributed to chronic inflammation and immune system dysfunction secondary to MT or low-level HIV-1 replication in plasma and cell reservoirs. Moreover, accelerated aging is significantly associated with chronic inflammation, immune activation, and immune senescence. In this review, we aimed to investigate the role of inflammation as a pivotal marker in the pathogenesis of HIV-1 disease. We will discuss the key features of chronic inflammation and immune activation that are observed during the natural course of the disease and those features that are detected in cART-modified infection. The review will focus on the following aspects of HIV-1 infection: (1) MT; (2) the role of residual viremia; and (3) "immune senescence" or "inflammaging." Many questions remain unanswered about the potential mechanisms that are involved in HIV-1 pathogenesis. Further studies are needed to better investigate the mechanisms that underlie immune activation and their correlation with HIV-1 disease progression.

Key words: Human immunodeficiency virus-1; Combined antiretroviral therapy; Immune activation; Microbial translocation; Residual viremia; Immune senescence

Core tip: The aim of this review was to summarize the most relevant mechanisms in human immunodeficiency virus-1 pathogenesis by focusing on the role of microbial translocation, residual viremia, and immune senescence or "inflammaging" in disease progression to full-blown AIDS. Moreover, the impact of antiretroviral therapy on these mechanisms was investigated.

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INTRODUCTION

Combined antiretroviral therapy (cART) has led to a

lower morbidity and mortality in human immunodeficiency virus type 1 (HIV-1)-infected patients by significantly improving clinical and laboratory parameters. However, the long-term use of cART is associated with adverse side effects that are generally not directly related to HIV-1 infection. These effects include cardiovascular diseases, kidney impairment, osteoporosis, and hepatotoxicities[1]. Moreover, the prolonged survival of patients and the persistence of virus particles in tissue may directly or indirectly contribute to the development of cancers, neurocognitive impairment and a more rapid progression of hepatitis C infection. Chronic inflammation, chronic immune activation, and immune senescence are the pathological hallmarks of HIV-1 infection that lead to these conditions in HIV-1-infected subjects, mainly in subjects with persistently decreased $CD4^+$ T cell counts. Cardiovascular events in HIV-1-infected patients may occur because of the following reasons: (1) these subjects have a higher cardiovascular risk than the general population; (2) the HIV-1 virus can increase the risk of atherosclerosis in patients; and (3) several antiretroviral regimens may influence the atherosclerotic profile of patients due to significant lipidic changes. Therefore, many ischemic cardiovascular events may occur during long-term HIV-1 infection and accelerated atherosclerotic processes may be related either to the infection or to the chronic use of cART[2]. Experimental studies have demonstrated the direct effect of several viral components on the endothelium[3], including the increased expression of adhesion molecules, such as intercellular adhesion molecule and E-selectin; a pro-thrombotic state with increased levels of von Willebrand factor, plasminogen activator inhibitor-1, and tissue plasminogen activator; leukocyte recruitment into the sub-endothelium; and atherosclerotic plaque growth[4,5].

Different factors may contribute to the establishment of immune activation during HIV-1 infection. HIV-1-specific mechanisms and non-specific generalized responses to infection may promote the chronic and aberrant activation of the immune system. An early loss of gut mucosal integrity, the pro-inflammatory cytokine milieu, co-infections, and the subsequent marked destruction of the lymph node architecture are the main factors that contribute to the ongoing activation of the innate and adaptive immune systems. The severe depletion of memory $CD4^+$ T cells, especially cells that express the CCR5 receptor, occurs in the gut mucosa during primary HIV-1 infection and simian immunodeficiency virus (SIV) infection[6].

A massive loss of mucosal T helper 17 (Th17) $CD4^+$ T cells in the SIV-infected rhesus macaque, an animal model of AIDS, has been linked to impaired immune responses in the gut mucosa to an enteric pathogen, which leads to the lack of local control of the pathogen and consequently its translocation[7]. Therefore, both the loss of immune mucosal function and the breakdown of the intestinal barrier may allow the translocation of microbial products into the systemic circulation. Findings suggest that plasma lipopolysaccharide (LPS) levels, which are used as a marker of microbial translocation (MT), are elevated during chronic HIV-1 infection[8]. Regarding cytokine imbalance patterns, higher levels of inflammation markers and coagulation factors, such as high-sensitivity C-reactive protein (h-PCR), D-dimer, and interleukin-6 (IL-6), have been observed in HIV-1-infected patients[9].

Overall, these changes in cytokine and coagulation profiles are associated with an increased risk of cardiovascular diseases, opportunistic conditions, and other mortality causes in subjects with $CD4^+$ T cell counts that are persistently below 500 cells/μL[10,11].

Considering the strong evidence that persistent immune activation is a key cause of HIV-1 disease progression, understanding the mechanisms that drive immune activation during chronic infection is important for developing new strategies that target this process.

CHRONIC IMMUNE ACTIVATION

The current simplified model of HIV-1 pathogenesis integrates the following three main events that occur during the natural or cART-modified course of viral infection: (1) the massive depletion of $CD4^+$ T lymphocytes; (2) paradoxical immune activation; and (3) the exhaustion of immune resources.

These events are briefly analyzed in the following paragraphs and are depicted in Figure 1: (1) During primary infection, HIV-1 can infect a large number of $CD4^+$ T cells, particularly the activated memory T cell subset that expresses the CCR5 ligand. This process is associated with high levels of viral replication[12]. The depletion of $CD4^+$ T cells that is observed in the setting of HIV/SIV (the simian equivalent of HIV) infection is due to the involvement of the central memory $CD4^+$ T cell population. Additionally, this event is based on the establishment of reservoirs of latently infected cells[13,14]. Studies in primates that were infected with SIV and in HIV-1-infected humans have revealed that massive $CD4^+$ T cell depletion occurs in mucosal tissue throughout all of the stages of HIV-1 infection[15]. Plasma HIV-1 viremia (the level of HIV-1 RNA in plasma) increases to peak levels until the adaptive immune response, particularly the onset of HIV-1-specific $CD8^+$ T cells, which generally indicates the end of the acute phase of infection. However, the damage to the immune system is significant: HIV-1 has established a latent reservoir and rooted itself in the host, and extensive viral replication has resulted in the massive depletion of $CD4^+$ T cells, especially in mucosal lymphoid tissue (MALT). Therefore, the compromised integrity of MALT may result in MT from the gut into the systemic circulation[16,17]; (2) HIV-1 infection is associated with chronic immune activation, which appears more pronounced in patients with an advanced cellular immunodeficiency[18,19]. This immune activation is charac-

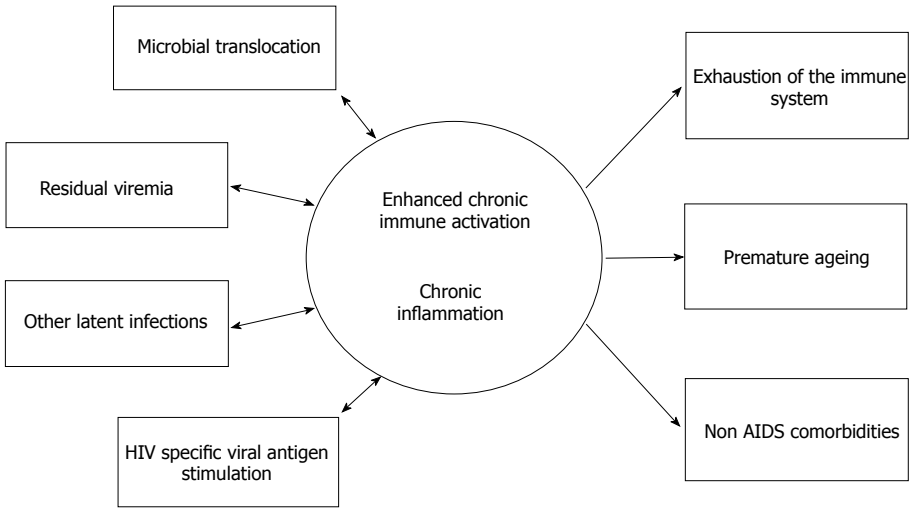

Figure 1 Factors associated with chronic inflammation and immune activation in human immunodeficiency virus-1 disease. HIV: Human immunodeficiency virus-1.

terized by the presence of chronically activated T cells, B cells and monocytes/macrophages; the increased expression of various leukocyte activation markers; the production of pro-inflammatory cytokines; and an increase in cell proliferation[20]. High levels of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), IL-6 and IL-1β, in both plasma and lymph nodes have been observed in the early stages of HIV-1 infection[21,22]. In addition, the secretion of chemokines, such as MIP-1α, MIP-1β and regulated upon activation, normal T cell expressed and presumably secreted (RANTES), is increased in these patients[23,24]. The persistent inflammation status is most likely due to several factors, including the ongoing production of HIV-1; the presence of co-pathogens, such as cytomegalovirus (CMV) or herpes viruses (HSVs); the translocation of LPS across a damaged gut mucosa; the loss of T regulatory lymphocytes and other immunoregulatory cells; and irreversible fibrosis of the thymus and the lymph node infrastructure. CMV causes life-long antigenic stimulation and the subsequent development of an expanded population of well-differentiated, apoptosis-resistant, senescent T cells with limited proliferative potential[25,26]. During HIV-1 infection, the depletion of $CD4^+$ T cells may result in the suboptimal immune control of these persistent viral infectious agents, which permits the reactivation and replication of CMV and Epstein-Barr virus (EBV) infections. Several authors have hypothesized that co-infections with other viruses may contribute to the "accelerated aging" syndrome that is observed in HIV-1 patient populations[27]. Therefore, this state of generalized chronic immune activation is currently considered the hallmark of pathogenic HIV-1 and SIV infections and has a higher independent predictive value of disease progression than viral replication[28]; and (3) During all of the stages of HIV-1 infection, the presence of strong and persistent immune activation is the primary cause of senescence and apoptosis of the immune system and ultimately leads to the exhaustion of immune resources. Immune activation and inflammation result in fibrosis of lymphatic tissue, which damages the lymph node architecture and prevents normal T cell homeostasis[29,30]. Moreover, a vicious cycle is established in which HIV-1 replication promotes immune activation and immune activation promotes HIV-1 replication. Pro-inflammatory cytokines are released and participate in this mechanism. The synergic action of IL-1β, TNF-α and IL-6 can lead to T cell activation. In addition, IL-1βand TNF-α may decrease trans-epithelial resistance in mucosal tissues[31,32]. cART has been considered the best "deactivator" of the immune system in HIV-1-infected patients. cART usually results in a marked reduction in T cell activation and apoptosis[33,34] and a decrease in pro-inflammatory cytokine levels. In addition, antigen-specific stimulation is strongly diminished due to the rapid decline in the number of HIV-1-specific $CD8^+$ T cells[35,36]. cART reduces the depletion of naïve T cells and induces immune recovery. However, even when a decrease in inflammation and the down-regulation of immune activation markers is observed in patients on cART, more inflammatory parameters remain at higher levels than those in healthy individuals and a significant imbalance in the cytokine profiles persists.

MT

The HIV-1-induced disruption of MALT results in the translocation of microbial products across the intestinal mucosa into the peripheral circulation, which produces high levels of plasma LPS and bacterial DNA that persist over time (Table 1). MT is correlated with markers of systemic immune activation[37]. LPS is a component of the cell wall of gram-negative bacteria, and the majority of authors suggest that LPS is a marker of MT throughout chronic HIV-1 infection[38].

Mucosal damage and the dysfunctional phagocytic clearance of microbial products are responsible for MT in the bloodstream. The translocation of bacterial

Table 1 Main dynamics involved in the development of immune dysfunction during human immunodeficiency virus-1 infection

Microbial translocation	Residual viremia	Immune senescence
HIV-1 invasion of the gut mucosa	MT is enhanced in patients presenting residual viremia	High frequency of $CD4^+CD38^+$ and $CD8^+CD38^+$ T cells
Disruption of mucosal integrity and depletion of local Th-17 cells	Stochastic antigen stimulation of long-lived latency infected cells	Accumulation of senescent antigen -experienced memory T
LPS, CpG DNA in blood stream with aspecific and polyclonal immune-activation *via* LPS	Viral replication in anatomical sanctuaries	Inefficient T cell renewal
Pro-inflammatory cytokines secretion (TNF-α; IL-1; IL-6)	Incomplete viral suppression during cART	Fibrosis of lymphopoietic organs cells ($CD28^-CD57^+$)

HIV-1: Human immunodeficiency virus-1; Th-17: T helper-17; LPS: Lipopolysaccharide; CpG: --C--phosphate--G--; TNF-α: Tumor necrosis factor-alpha; IL-1: Interleukin-1; MT: Microbial translocation; cART: Combined antiretroviral therapy.

products results in the profound activation of the innate immune response. LPS, flagellin and CpG DNA, which are toll-like receptor (TLR) ligands, can directly stimulate peripheral macrophages and dendritic cells to produce a range of pro-inflammatory cytokines. Several investigations have demonstrated that LPS is biologically active *in vivo* and its interaction with CD14/TLR-4 on monocyte/macrophages is one of the mechanisms that leads to the secretion of soluble CD14 (sCD14) and pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1. SCD14 is produced by monocyte/macrophages in response to stimulation by LPS. LPS stimulation in vitro has been demonstrated to promote T lymphocyte activation and death[39,40].

The correlation between plasma LPS levels and the frequency of circulating $CD8^+$ T cells with an activated $CD38^+$ HLA-DR+ phenotype suggests that MT may directly or indirectly generate polyclonal T cell activation *via* the production of cytokines and chemokines[41].

Consistent with these observations, subjects with HIV-1 infection and high levels of LPS have an increased risk of disease progression to full-blown AIDS or death, irrespective of their $CD4^+$ T cell counts and HIV-1 RNA levels viral load (VL). Moreover, this marker has been demonstrated to be a strong predictor of mortality, independent of the $CD4^+$ T cell count and the VL[42,43].

The translocation of bacterial products into the systemic circulation through the damaged gastrointestinal barrier has been described as a pivotal driver of immune activation in the course of chronic HIV-1 infection[44-47]. MT is the result of $CD4^+$ T cell depletion in the gut mucosa and increased gut permeability; however, MT has been observed in other diseases, such as idiopathic $CD4^+$ T cell lymphocytopenia[48]. High levels of MT have been observed in many HIV-1-naïve patients. Several studies suggested that cART induces a progressive decrease in the plasma levels of microbial DNA, which tends to stabilize after several weeks of treatment but never normalizes[44]. A reduction in MT and inflammatory markers is broadly associated with a decrease in HIV-1 load. Moreover, recent findings have indicated that the presence of MT is associated with residual viral replication in HIV-1-infected subjects who receive effective cART. Those subjects with higher viral suppression (*i.e.*, VL < 2.5 copies/mL) presented the same LPS levels as HIV-1-uninfected subjects, which suggests that cART may have reverted HIV-1-induced mucosal damage[49]. However, other authors found that MT is strongly associated with higher levels of inflammation markers, independent of HIV-1 VL levels. Despite the findings that cART can reduce MT levels, inflammatory marker levels remain higher than those observed among uninfected subjects[50]. Recent findings in cART-treated subjects revealed that HIV-1 DNA levels in the gut mucosa were strictly correlated with LPS levels and the number of $CD8^+CD38^+$ T cells[51].

Long-term cART is associated with reduced plasma LPS levels and the down-regulation of immune activation markers. However, LPS plasma levels often remain detectable in patients who are successfully treated with cART[44]. This phenomenon may be explained by the ongoing partial repair of the mucosal barrier during cART. The LPS levels in subjects with maximal viral suppression are comparable to those observed in healthy donors. However, the mechanisms of LPS reduction after starting cART are not well understood because these mechanisms do not depend on VL but come into play soon after treatment initiation. However, the lack of an association between reduced MT and increased $CD4^+$ T cells during the first weeks of cART suggests that MT is more influenced by the cellular turnover of latently infected cells than from circulating $CD4^+$ T-cells.

The following questions regarding the role of MT in HIV-1 pathogenesis remain unsolved: (1) During the natural course of HIV-1 disease, does MT contribute to immune system activation or is MT a consequence of immune system activation? (2) Is MT the sole cause of immune activation in HIV-1-infected patients or does residual viremia play a pivotal role? If yes, what is the importance of these two mechanisms? (3) Is gut mucosal damage completely reversible after starting cART? and (4) Moreover, does LPS play a key role in virologically controlled patients with blunted $CD4^+$ T cell gain?

RESIDUAL VIREMIA

The objective of cART is to maintain plasma virologi-

cal suppression below the limits of detection, which are generally less than 50 copies/mL depending on the assay that is used[52]. Several studies have demonstrated that maintaining viral load levels < 50 copies/mL leads to long-term virological success and immunological and clinical benefits in HIV-1-infected subjects. However, the main methods that are used to evaluate HIV-1 RNA load during HIV-1 infection have various detection limits. The polymerase chain reaction (PCR) assay has a detection limit of 400 copies/mL. The ultrasensitive PCR assay has a detection limit of 50 copies/mL, and the real-time PCR assay has a lower limit of detection that ranges from 20-48 copies/mL[53]. The lower limits of detection of the new real-time assays may result in increased measurements of transient and intermittent detectable viral RNA (blips) in patients with virological suppression. There is controversy about the significance and consequences of viral blips. Several authors suggest an association between blips and the development of mutations that confer resistance to cART and an increased risk of virological failure[54-56]. In contrast, other authors did not find any relationship between isolated blips and virological failure[57,58]. Intermittent viremia increases T cell activation and facilitates the extension of HIV-1 infection. Subjects with intermittent viremia present higher levels of total specific $CD8^+$ and $CD4^+$ T cell responses compared with patients who have persistently undetectable HIV-1 RNA levels. These $CD8^+$ and $CD4^+$ T cell responses may block viral replication, thereby reducing the risk of virological failure[59]. The discrepancies in the findings may be due to inconsistencies in the definitions of blips and virological failure and to differences in the testing methods for the detection of HIV-1 RNA levels[60].

Recent studies have used a laboratory-based real-time PCR assay that was capable of detecting single HIV-1 RNA copies/mL. These studies demonstrated that several patients who received cART had persistent low-level viremia that ranged from 1-49 copies/mL. The source and dynamics of persistent viremia in treated patients are currently under investigation. It has been proposed that low-level viremia may be the result of ongoing viral replication in patients, which is caused by incomplete viral suppression during cART[61]. Therefore, several studies have investigated whether intensification with raltegravir, an integrase inhibitor that blocks viral DNA integration into host cell DNA, would further decrease the persistent low-level residual plasma viremia in patients on effective cART[62]. In subjects who were treated during chronic infection, the intensification of cART with raltegravir for 48 wk was associated with a significant decrease in $CD8^+$ T cell activation and a transient increase in episomal HIV-1 DNA, which suggests that raltegravir intensification may positively impact residual HIV-1 replication[63].

The absence of any detectable effects of drug intensification on HIV-1 residual viremia in patients on therapy suggests that viremia is not due to ongoing replication but may arise from different sources. An alternative hypothesis is that the residual amount of HIV-1 RNA may be the result of virus release from long-lived latently infected cells that are activated by stochastic antigen stimulation (Table 1). Several papers have reported that genetically homogeneous viral subpopulations can often be observed in patients on long-term treatment and in the viral population that rebounds during treatment interruptions. These findings further support the concept that persistent low-level viremia arises from long-lived cells rather than ongoing viral replication[64,65].

A further line of investigation has focused on anatomical compartments that may serve as "sanctuary sites", such as the central nervous system and the genital tract, in which HIV-1 replication can occur unhindered by poorly penetrating antiretroviral agents. However, the role of ongoing HIV-1 replication in tissue compartments and cellular reservoirs remains to be defined. Several findings suggest that the reservoir is mainly established and maintained in tissue and that infected cells that are circulating in the blood may not be representative of the much larger population of infected cells in tissue. Sequences of persistent HIV-1 populations in plasma are often not found in peripheral blood resting memory $CD4^+$ T cells[61-65]. Understanding the relationship between residual low-level viremia and the size of the reservoir will help guide future attempts at HIV-1 eradication; however, further prospective studies are required to determine the cause-and-effect relationship between these parameters.

Several studies that have used conventional HIV-1 RNA assays suggest that a chronic inflammation status may persist in patients with undetectable HIV-1 RNA loads[53,66]. The persistency of low-level residual viremia represents a continuous pro-inflammatory stimulus for the immune system, which underlies chronic immune activation and inflammation. Chronic inflammation and immune system dysfunction are important contributors to the increased risk of non-AIDS comorbidities that are often observed in HIV-1 patients, such as cardiovascular events, renal impairment and non-AIDS cancers[41,67]. Moreover, increased levels of inflammation have been associated with an increased risk of progression to AIDS and mortality in HIV-1 patients. In contrast, viremia control is accompanied by a decrease in MT, chronic inflammation and immune system activation parameters[68]. Whether residual low-level viremia plays a key role in increasing the inflammatory status in patients is unclear, and different results have been reported. In the SMART trial, markers of inflammation, coagulation and renal function were elevated in HIV-1 participants and remained elevated even after HIV-1 RNA levels were suppressed with cART[69].

Elite controllers have higher levels of the inflammatory marker C-reactive protein (CRP) than uninfected controls. This finding may be explained by the presence of infected $CD4^+$ T cells that carry replication-competent HIV-1 particles, which suggests that low levels of

ongoing viral replication contribute to the maintenance of HIV-1 reservoirs in the absence of detectable plasma viremia[70]. However, no association has been found between low-level viremia and CRP, fibrinogen and IL-6 levels, which suggests that CRP may not be a reliable marker of inflammation due to ongoing viral replication or viral persistence. In addition, no correlation has been found between immune activation markers and residual viremia[61,66,68]. HIV-1-infected patients with high levels of LPS have an increased risk of progression to AIDS. Plasma LPS levels are correlated with the persistence of HIV-1 in the gut mucosa. Furthermore, HIV-1 DNA levels are correlated with the levels of the activation marker CD38 and $CD8^+$ T cell numbers. Recent studies found that HIV-1-infected patients on cART who had negative HIV-1 RNA plasma levels (< 20 copies/mL) presented less frequently with MT and had lower levels of inflammation markers than patients with low-level viremia (20-200 copies/mL), which suggests that inflammation is induced by MT and not by HIV-1 viremia[50].

These contrasting data indicate that the mechanisms by which residual viremia and chronic inflammation increase the risk of morbidities and mortality in HIV-1-infected subjects on cART are complex, and further studies are needed.

HIV-1 AND IMMUNE SENESCENCE

The association between HIV-1 infection and inflammation is similar to that between advanced age and inflammation, which has been well described. HIV-1 infection shares several similarities with aging, including an increased incidence of cardiovascular diseases, malignancies, infections, chronic viral reactivations, osteoporosis, neurocognitive decline, and frailty[1,71].

Similar to aging, HIV-1 infection is characterized by a general decline in T cell renewal, and an altered capability to regenerate T lymphocytes has been observed in both conditions. Therefore, the naïve T cell pool cannot be efficiently replenished and old, exhausted $CD8^+$ T cell clones and depleted $CD4^+$ T cells cannot be continuously replaced. The double insult of aging and HIV-1 infection impacts the functions of both the hematopoietic stem cell compartment and the thymus and may contribute to many of the changes that are associated with immune senescence, including reduced naïve T cell production, reduced T cell proliferation, and an impaired immune system response to vaccines and infections. The direct infection of the thymic stroma and thymocytes by HIV-1[72-74] and the thymic atrophy that is observed in HIV-1-infected subjects may account for this immune decline, which is similar to age-related thymic involution[75] and may be the result of the suppressive effects of pro-inflammatory cytokines on the thymus[76].

In both aging and HIV-1 infection, the increased expression of the activation marker CD38, which is expressed on the surface of $CD4^+$ and $CD8^+$ T cells, has been observed[77,78]. Moreover, positive correlations have been observed among the proportion of $CD8^+$ T cells that share the HLA-DR+/$CD38^+$ phenotype, the rate of $CD4^+$ T cell decay and the development of opportunistic diseases[79,80]. In addition, persistent T cell activation leads to T cell proliferation and T cell differentiation, which results in the accumulation of senescent, antigen-experienced memory T cells, the reduced expression of CD28 and an increased expression of CD57[81].

The expression of the surface marker CD57 has been correlated with greater resistance to apoptosis in $CD8^+$ T lymphocytes during HIV-1 infection, which facilitates T cell accumulation[82]. CD28 is a co-stimulatory molecule, and the loss of this marker on $CD4^+$ and $CD8^+$ T cells results in reduced B cell function and restricted T cell diversity. A high proportion of $CD8^+$ T cells that express CD57 has been observed in both aging and HIV-1 infection, and this senescent $CD28^-$/$CD57^+$ phenotype is characterized by a reduced capacity to produce IL-2 and a shortened telomere[83,84]. A higher frequency of senescent $CD8^+$ T cells ($CD45RO^+CD57^+CD28^-$) and a lower frequency of naïve $CD4^+$ and $CD8^+$ T cells ($CD45RA^+CD28^+CCR7^+$) were found both in cART-treated patients with undetectable viremia and high $CD4^+$ T cell counts and in older HIV-negative individuals when compared with HIV-1-negative younger controls. The expression of $CD8^+$ T cell activation markers (HLA-DR+$CD38^+$) was higher in HIV-1-infected individuals than in older or younger seronegative individuals[85] (Table 1).

The disproportionate production and accumulation of cytokines, such as TNF-α, IL-1β and IL-6, may lead to several adverse effects. Pro-inflammatory cytokines share a pivotal role in the process of aging and are present at higher concentrations in the blood of the elderly[86,87]. IL-6 is directly associated with the development of age-related disorders, including osteoporosis, cognitive decline and frailty symptoms, whereas increased plasma levels of TNF-α and IL-1β have been observed in the elderly with atherosclerosis[88-90]. In addition, these cytokines may have a role in neurocognitive impairment and neuronal[91-93] pathologies most likely through the induction of large amounts of nitric oxide[94-96], which is conducive to oxidative stress-related damage. This overall process can be referred to as "inflammaging", which is the up-regulation of anti-stress responses and inflammatory cytokines[97]. During the chronic phase of HIV-1 infection, both the accelerated process of immune senescence and inflammaging may contribute to the development of the progressive immunodeficiency.

CONCLUSION

Many questions remain unanswered about the mechanisms that underlie HIV-1 pathogenesis and the role of microbial product translocation, residual viremia and immune senescence in the development of persistent

immune activation and chronic inflammation, which is present at different degrees in all HIV-1-infected subjects. Moreover, despite effective cART-mediated viral suppression, persistent immune activation and inflammation have emerged as a major problem in the current HIV-1 era. Chronic inflammation and persistent immune activation remain abnormally elevated in many HIV-1-infected individuals and can be used to predict disease progression, subsequent mortality and non-AIDS-related morbidities, including cardiovascular diseases.

Different studies have linked inflammatory indexes, cytokine networks, and immune activation markers to clinical outcomes, which validate persistent immune activation as a possible therapeutic target. Other recent investigations have helped to elucidate the role of residual viremia, MT, and immune senescence in driving this persistent inflammatory state. These findings may contribute to the identification of new targets for novel intervention strategies that are aimed at minimizing immune activation and inflammation, such as anti-inflammatory molecules, *i.e.*, corticosteroids, cyclosporine, hydroxychloroquine, aspirin, omega-3 fatty acids, vitamin D and statins. Other interventions, such as IL-2 and IL-7 treatments, may be useful to restore the regenerative capacities of the immune system and to reconstitute the thymic microenvironment and the production of naïve T cells. Experimental strategies that have demonstrated promising anti-aging effects include the use of resveratrol, rapamycin, acetyl-L-carnitine, alpha-lipoic acid, telomerase activators, caloric restriction and stem cell therapy[98].

The effective monitoring of HIV-1-infected patients requires the evaluation of activation biomarkers in clinical practice, which may help guide treatment decisions and may be used to better characterize the infection stage and the risk of disease progression.

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REVIEW

Is there an unrecognised role for *Campylobacter* infections in (chronic) inflammatory diseases?

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Abstract

Campylobacter species are one of the major causes of global bacterial-related diarrheal disease worldwide. The disease is most frequently associated with the ingestion of contaminated meat, raw milk, pets, contaminated water, and the organism may be frequently cultured from the faeces of chicken and other domesticated farm animals. Of the 17 established *Campylobacter* species, the most important pathogens for humans are *Campylobacter jejuni* (*C. jejuni*), *Campylobacter coli* (*C. coli*) and *Campylobacter fetus* (*C. fetus*), which are all associated with diarrheal disease. Further, *C. jejuni* and *C. coli* are also associated with the neuroparalytic diseases Guillain-Barré syndrome and Miller Fischer syndrome, respectively, whereas *C. fetus* is linked with psoriatic arthritis. The discovery of both "molecular mimicry" and translocation-related virulence in the pathogenesis of *C. jejuni*-induced disease, indicates that *Campylobacter*-related gastrointestinal infections may not only generate localized, acute intestinal infection in the human host, but may also be involved in the establishment of chronic inflammatory diseases. Indeed, pathogenicity studies on several *Campylobacter* species now suggest that molecular mimicry and translocation-related virulence is not only related to *C. jejuni*, but may play a role in human disease caused by other *Campylobacter* spp. In this review, the authors provide a review based on the current literature describing the potential links between *Campylobacter* spp. and (chronic) inflammatory diseases, and provide their opinions on the likely role of *Campylobacter* in such diseases.

Key words: *Campylobacter* spp; Infection; Autoimmune diseases; Chronic diseases

Core tip: *Campylobacter* species are able to induce both gastrointestinal and systemic infections in humans and have been linked not only to acute disease, but also to a wide range of (chronic) inflammatory diseases. In this respect, the organism is particularly associated with inflammatory peripheral nerve disease Guillain-Barré syndrome and reactive arthritis. However, the true role of *Campylobacter* in other human inflammatory diseases remains to be determined. This review indicates that the actual role of *Campylobacter* in human inflammatory diseases may be largely underestimated and suggests that further research is necessary in order to accurately determine the importance of *Campylobacter* infection in these diseases.

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INTRODUCTION

Campylobacter species are Gram negative zoonotic hu-

man pathogens that are one of the major causes of global bacterial-related diarrheal disease worldwide, a disease most frequently associated with the ingestion of contaminated animal products such as chicken meat, raw milk, contaminated water, and contaminated farm animals. Currently, of the 17 established *Campylobacter* species the most important associated with human disease is *Campylobacter jejuni* (*C. jejuni*), a leading cause of diarrheal disease worldwide with 400-500 million laboratory confirmed cases each year[1]. Further, this species can be sub-divided into two separate groups based on the presence or absence of sialic acid components attached to carbohydrate residues present on the bacterial outer surface[2]. The transfer of these sialic acid components to the carbohydrate outer surface of *C. jejuni* is mediated by the enzymes sialyltransferase Cst-Ⅱ or Cst-Ⅲ[3], with the presence of sialic acid conferring a highly pathogenic phenotype to the bacterium that has the potential to cause severe colitis[4], as well as paralytic disease. One such paralytic disease is Guillain-Barré syndrome (GBS), a post-infectious life threatening complication often associated with *C. jejuni* infection[5]. In fact, evidence suggests that GBS is facilitated by bacteria-human cross-reactive antibodies, generated *via* a process called "molecular mimicry". Essentially, some sialylated carbohydrate lipooligosaccharide (LOS) structures on the *C. jejuni* outer membrane possess epitopes that appear similar to certain ganglioside epitopes present on human peripheral nerves. This similarity may result in the production of auto-antibodies that target not only the bacterium, but also human nerves, inducing complement-mediated nerve destruction[5]. Further research has also shown that *C. jejuni* strains possessing sialylated LOS structures are significantly more invasive than non-sialylated strains, and are also better able to translocate across the intestinal epithelium[6-8]. In this respect, the authors have previously suggested that infection with *C. jejuni* and other *Campylobacter* spp. may actually be linked with a significant number of undetected bacteremias[9], and that the detection of *Campylobacter* species in current blood culture systems might be underrepresented, not least because these systems are not optimized for the special growth requirements of this bacterial generus[10].

Interestingly, Houliston *et al*[11] established that *Campylobacter* spp. are able to synthesize a much broader range of human mimicking glycolipid/glycoprotein structures in their lipopolysaccharides (LPS) and LOS than previously thought, *i.e*., *Campylobacter* species are equipped with a set of LPS/LOS genes that allow adaptation to their host, possibly allowing the organism to "hide" from recognition by the host immune system. In this hypothesis, LPS or LOS epitopes that mimic host antigens are expressed on the surface of *Campylobacter* bacteria in order to provide protection against the host immune response (*Campylobacter* antigens being recognized as self-antigens and therefore being less likely to be recognized by the host). However, this type of *Campylobacter* LPS and LOS molecular mimicry could potentially be a trigger for the development of as yet unrecognised inflammatory disease states in susceptible hosts. For example, there already exists many publications describing the role of *C. jejuni* in the aetiology of inflammatory diseases such as GBS and Miller Fischer syndrome (MFS), and the reader is referred to[5] for a recent review on this subject.

Worryingly, there are indications that healthy people may actually be (chronic) carriers of *Campylobacter* bacteria, again suggesting that this bacterium is able to adapt itself to the human host and escape immune recognition[12,13]. To confirm these observations, well designed surveillance programs are required in order to identify whether apparently healthy humans can be carriers of *Campylobacter* bacteria[14]. Although the carrier state of *Campylobacter* is not completely clear for humans, it has already been established that various animal species can act as carriers of *Campylobacter* spp. without displaying symptoms[15-19], and animal carriers have been linked to the induction of Campybacteriosis in humans[20-22]. Acute infections with *Campylobacter* species *via* food products, water or animals may also lead to chronic infections in humans[23,24], specifically when patients are suffering from an immunodeficiency[25-28].

In this review, the authors describe and comment on the current literature regarding the potential role of *Campylobacter* spp. in human (chronic) inflammatory diseases. The authors concentrate first on those immunologically-related diseases where a strong association between *Campylobacter* infection and disease has been shown, and then highlight those diseases where an association with *Campylobacter* infection is weaker, but where further research may be warranted. The authors conclude that there is indeed a potential role for *Campylobacter* spp. in the induction of many different types of (chronic) inflammatory diseases, and that this is most likely related to the link between *Campylobacter* infection, inflammation and molecular mimicry.

GBS AND MF SYNDROME

GBS and MF syndromes are (sub)acute inflammatory polyradiculoneuropathies affecting the peripheral nerves of affected patients[5]. GBS and MF patients experience degeneration and demyelination of specific neuronal axons after an episode of gastrointestinal or respiratory infection, with demyelination being triggered by an autoimmune-like response[5]. In GBS patients the muscles in the body become paralysed, whereas in MF patients, only the facial muscles are affected[5].

GBS is also called the Landry-Guillain-Barré-Strohl syndrome and is named after the four scientists that originally discovered and reported on this disease[29]. In 1859 Landry de Thézillat was the first to describe an ascending paralyzing disease in great detail[30], although earlier publications mimicking the disorder of Landry were reported by Auguste François Chomel (1788-1858) in 1828[31] and James Wardrop (1782-1869) in 1834[32]. In 1916 Guillain *et al*[33] reported on an examination of

two soldiers that were suffering from muscular weakness, paresthesias, and muscular pain. In 1927, Draganesco *et al*[34] defined the nomenclature "Guillain-Barré syndrome" to describe this paralyzing disease. In 1982, Rhodes *et al*[35] reported for the first time that the GBS syndrome was associated with *Campylobacter* infection, which was later confirmed by Constant *et al*[36] and Speed *et al*[37]. In all of these studies, it was recognized that diarrhoea often preceded the appearance of Guillain-Barré syndrome, with later bacterial culture and serum diagnostic tests revealing that *C. jejuni* was often the causative agent of the intestinal infection that preceded the onset of the GBS syndrome[38-45].

In 1990, it was suggested for the first time that *C. jejuni* might stimulate the production of antibodies against the myelin sheet of the peripheral nerves of GBS patients[45]. In this same year, Yuki *et al*[46], demonstrated that 2 GBS patients possessed high serum IgG titres against GM1 ganglioside following *C. jejuni* enteritis, a significant finding as these results greatly accelerated the research on *C. jejuni*-induced GBS. Subsequently, multiple research groups confirmed the work by Yuki *et al*[46], linking anti-ganglioside antibodies with *C. jejuni*-induced GBS[47-50]. Eventually, other research groups established that certain *C. jejuni* serotypes (Penner O:4, O:19 and O:41) were more frequently isolated from GBS patients than from enteritis patients, and that anti-GQ1 antibodies were linked to the onset of Miller Fisher syndrome[51-59]. The results of these findings lead to the hypothesis that there exists a form of "molecular mimicry" between *C. jejuni* cell envelope structures and ganglioside structures present on the peripheral nerves of affected patients, suggesting that an immunopathogenic mechanism might be causing damage to the nerves in GBS and MFS disease[60,61]. Indeed, Goodyear *et al*[62], not only showed that monoclonal antibodies raised against the LOS isolated from GBS-inducing *C. jejuni* strains reacted with ganglioside structures on the peripheral nerves, but that they possess the potential to block muscle-nerve interactions. The subsequent finding that *C. jejuni* possesses genes that enable the sialylation of its outer membrane LOS further increased the suspicion that molecular mimicry might form the basis for *C. jejuni*-induced GBS[63-65]. Though mainly associated with *C. jejuni*, research has indicated that molecular mimicry may play roles in human infections associated with several other *Campylobacter* species, particularly with *C. coli*[38,41,66,67].

Based on these findings, there is the potential for an as yet undescribed *Campylobacter*-human autoimmune interactions at the level of molecular mimicry. Further, these interactions could provide the basis for a range of, as yet undefined, chronic and acute inflammatory *Campylobacter*-associated diseases in humans.

INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) is a term used for a group of chronic inflammatory disorders of the gut that includes two major diseases. These are Crohn's disease (CD) and ulcerative colitis (diseases associated with alteration of the ileum, colon and rectum leading to cramps, fever and bloody diarrhoea). Currently, the association between *C. jejuni* infection and chronic IBDs such as CD is controversial[25,68]. During a four-year study in 1982 in Belgium, 45 patients were found to exhibit signs of an acute infective colitis. Twenty percent of these patients were found to be positive for *C. jejuni,* the bacterium found to be associated with focal colitis, necessitating a differential diagnosis from Crohn's colitis[69]. During the same period, 12 patients were also admitted with an acute attack of IBD due to an intercurrent infection with bacterial pathogens including *C. jejuni*[69]. These data indicate that, in the presence of an acute attack of colitis, an infective etiologic agent should always be sought, and that an attack of chronic idiopathic IBD may be caused by an intercurrent infection. van Spreeuwel *et al*[70] compared 22 patients with *C. jejuni*-induced colitis against 10 healthy controls, 10 ulcerative colitis patients and 10 CD patients, and concluded from immunological analyses that *C. jejuni* colitis can be distinguished from ulcerative colitis and CD patients by IgG containing plasma cells. Also, around the same time, Simson *et al*[71] found that *C. jejuni* was associated with acute relapse and abscess formation in CD. In a Bulgarian study, Boyanova *et al*[24] analyzed the frequency of *Campylobacter* species isolation from patients with acute enterocolitis, IBD, and other chronic intestinal diseases. The authors screened 682 Bulgarian patients and established that *Campylobacter* species were detected in patients with acute enterocolitis (7.8%), chronic enterocolitis (6.2%), CD (6.2%), ulcerative colitis (3.7%), and irritable bowel syndrome (8.3%)[24]. Further, hippurate-positive *C. jejuni* isolates accounted for 62.2% of *Campylobacter* strains. The authors concluded that *Campylobacter* could be one of the causes of chronic intestinal diseases in Bulgaria[24]. Interestingly, Berberian *et al*[72] (1994), showed that the expression of a novel autoantibody defined by the *VH3-15* gene could be detected in both IBD and *C. jejuni* enterocolitis patients. These authors screened 101 individuals with ulcerative colitis, CD or other acute or chronic colitis symptoms. Compared with normal subjects, $BK2^+$ anti-erythrocyte Abs were elevated in most sera from patients with CD and ulcerative colitis (including post-colectomy)[72]. However, $BK2^+$ anti-erythrocyte antibodies were also elevated in 10 of 38 non-IBD patients, all of whom had *C. jejuni* enterocolitis[72]. The findings by Berberian *et al*[72] tended to suggest that a common immunopathogenetic factor, manifested by VH3-15 B cell activation, may be shared between ulcerative colitis, CD, and *C. jejuni* enterocolitis. An indirect effect of *C. jejuni* in the aetiology of IBD was suggested by several different studies of Kalischuk *et al*[73], who established that transcytosis of *C. jejuni* across gut epithelia allowed other commensal gut flora to also cross the intestinal epithelial barrier[68,73,74]. Apparently, the translocation of commensal flora may result in an inflammatory immune response against the commensal gut flora (luminal antigen translocation hypothesis),

a process that is commonly observed in patients suffering from IBD[75,76]. With respect to population studies, Gradel *et al*[77] (2009) compared 13148 people from 2 Danish counties who had been exposed to *Salmonella* and *Campylobacter* gastroenteritis, with 26216 unexposed individuals. After an average follow-up of 7.5 years, the hazard ratio of first-time IBD diagnosis was 2.9 (exposed to unexposed), and was raised for both CD and ulcerative colitis[77]. In those exposed to *Campylobacter* and *Salmonella* only 1.2% of the studied subjects developed IBD. Thus, while the study identified bacterial exposure as a statistically significant factor in IBD the role of *Campylobacter* seems to be only of relative importance. Following the Danish population, but in a different study (1992-2008), Jess *et al*[78] showed that infection with *Campylobacter* species, confirmed by *Campylobacter* isolation from stool samples, significantly increased the risk of developing IBD. However, contradicting this observation, was the finding that culture negative stool samples were also significantly associated with an increased risk for the development of IBD[78]. The final conclusion of the authors was that the increased risk of IBD after infections with intestinal pathogens might be a result of a detection bias, due to increased testing for such pathogens in this patient group[78]. Subsequently, Riddle *et al*[79] further discussed this conclusion stating that due to study limitations and diagnostic bias there could in fact be several different explanations for finding an association between culture positivity or culture negativity in IBD patients.

A more direct link between a *Campylobacter* species (not *jejuni*), CD and IBD was observed more recently. In 2009-2011, Zhang *et al*[80] were able to link the presence of *Campylobacter concisus* (*C. concisus*) to pediatric CD using the techniques of polymerase chain reaction (PCR) bacterial detection and the presence of specific IgG antibody, an observation that has also been associated with adults presenting with IBD[80-82]. Further, in 2011, Kovach *et al*[83] identified actual *C. concisus* proteins that were immunoreactive within patients with CD.

However, not all studies have been successful in linking *C. jejuni* (or any other *Campylobacter* species) with IBDs. In 1984, Blaser *et al*[84] studied 72 CD patients using culture, serology and immunohistochemistry, and concluded that *C. jejuni* was not likely to be an etiological agent of CD or chronic ulcerative colitis. In a Scandinavian study using 95 patients with *Campylobacteriosis,* it was observed that 77%-86% patients harbored a raised antibody titre against an antigen mixture comprising seven *C. jejuni/coli* strains including a PEN 0:6, 7 isolate, which represented the most common serotype in Scandinavia[85]. In this same study, the authors also analyzed the sera of 56 IBD patients and found that none reacted with this *C. jejuni/coli* antigen mixture and concluded that *Campylobacteriosis* was not associated with these chronic diseases[85]. In 1992, a prospective study began by analyzing 64 IBD patients 15 of whom were diagnosed with ulcerative colitis. Stool samples were screened for enteric pathogens, but only a low number of these samples were confirmed to be culture positive. The conclusion of this study was that enteric microorganisms including *C. jejuni* only play a minor or indeed a negligible role in the exacerbation of IBD[86]. Therefore, the link between *C. jejuni* and IBD seems to be weak, but based on current literature, *C. concisus* might by *Campylobacter* species harboring more potential to be a causative agent of IBD.

IRRITABLE BOWEL SYNDROME

Irritable bowel syndrome (IBS) differs from IBD in the fact that IBS is a collection of gut-related disease signs and symptoms with no known aetiological cause. Though they have several symptoms in common, treatments for IBS and IBD vary greatly. Only a minority of IBS reported disease is associated with a post-infection process (PI-IBS)[87], though one of the commonest causes of PI-IBS appears to be *C. jejuni* infection[88]. The main mechanism for inducing *C. jejuni*-related IBS appearing to be the production of cytolethal distending toxin (CDT) by *C. jejuni*[89]. However, experiments in a rat model have indicated that histopathological changes in the gut during *C. jejuni* infection may be caused by both CDT-producing and non-producing isolates[90]. Therefore the role of *C. jejuni* CDT in IBS remains a point of discussion. One other mechanism involved in the aetiology of IBS is the promotion of inflammation of the gut tissue *via* the generation of a "low grade" immune response (involving autoendocrine cells, CD3, CD4 and CD8 lymphocytes) and gut permeability[91].

Recently, it has been suggested that blockage of the PI3K-γ signalling pathway in *Campylobacter* infection may be a means of reducing severe inflammation facilitated by the innate immune system[92]. Indeed, treatment of *C. jejuni* infection with the antibiotic rifamixin in a rat model of IBS infection stopped the development of long-term altered stool function and form (a phenomenon linked to the overgrowth of the small intestine with *C. jejuni* bacteria, and a characteristic IBS-associated phenotype)[93].

REACTIVE ARTHRITIS (REITER'S SYNDROME)

Reactive arthritis is an inflammation of the joints which develops whilst suffering/recovering from a recent infection. Though other symptoms also usually develop in addition to arthritis, joint inflammation is the main characteristic of this disease. Any site of infection may be associated with reactive arthritis, including the intestine (the site of infection for *C. jejuni*)[94], with symptoms commonly lasting 3-12 mo, though in some cases, the arthritis may persist long-term[94]. Reiter's syndrome a variant of reactive arthritis is established when the following symptoms occur simultaneously; urethritis, arthritis and conjunctivitis[94].

In 1979, a case report was published that linked a *C. jejuni* infection with the induction of reactive arthritis for the first time[95]. In this case report, reactive arthritis de-

veloped two weeks after the subject experienced watery diarrhoea containing blood, and was experiencing anorexia, and severe weight loss[95]. The causative agent of the infection was found to be *C. jejuni*[95]. In a later study, it was established that reactive arthritis was more likely to occur in *C. jejuni* enteritis patients that were positive for histocompatibility antigen HLA-B27[96], and around the same time period different groups more or less confirmed this finding[97-99]. Importantly, HLA-B27-negative arthritis-related *C. jejuni* enteritis cases are nevertheless sporadically reported[97,100-105]. Interestingly, patients presenting with ankylosing spondylitis (a chronic inflammatory disease) overwhelmingly possess HLA-B27 and molecular mimicry with the gut bacterium *Klebsiella pneumoniae* is thought to play a key role in disease development[106,107]. However, research has indicated that there are no signs of *C. jejuni*/*C. coli*-related antibodies in patients with active ankylosing spondylitis[108]. More recently, Mortensen *et al*[4] was able to link a potential virulence factor, namely class A sialylated lipooligosaccharide structures, to a more severe gastro-enteritis phenotype and reactive arthritis, suggesting that sialylated LOS structures, structures that mimic human gangliosides, are also a risk factor in the development of reactive arthritis. Interestingly, the possession/expression of reactive arthritis-related sialylated LOS structures does not appear to be related to any particular *C. jejuni* genotype[109]. For further information, the reader is referred to a systematic review by Pope *et al*[110], which summarizes the link between *Campylobacter* spp. and reactive arthritis.

Up to this point, there has been strong evidence for an association between *Campylobacter* infection and a range of (chronic) inflammatory diseases, GBS, MF, IBD and IBS. However, in the following section, the authors discuss diseases where the link between *Campylobacter* infection and (chronic) inflammatory disease is much weaker. In this respect, the authors would like to see more research in this area, in order to finally confirm or deny any unrecognised association between *Campylobacter* infection and the following diseases.

SYSTEMIC LUPUS ERYTHAMATOSUS

Systemic lupus erythematosus, often abbreviated to SLE or lupus, is a systemic autoimmune disease that can affect any part of the body[111]. As with other autoimmune diseases, the immune system attacks its own cells and tissues, resulting in inflammation and tissue damage[111]. Lupus most often affects the heart, joints, skin, lungs, blood vessels, liver, kidneys, and nervous system[111]. The course of the disease is unpredictable, with periods of illness (flares) alternating with remissions. The disease occurs nine times more often in women than in men, mainly in women in the child-bearing years of 15 to 35, and is also more common in those of non-European descent[111]. Currently, over a 100 articles linking SLE with the GBS have been published, one of the main causative agents of GBS being *C. jejuni*[5]. As early as 1984, Johnson *et al*[112] described a persistent *C. jejuni* infection in a patient with lupus and a deficiency in serum IgA and IgM. The authors showed that the serum of this patient was not able to kill the *C. jejuni* bacterium. In 1998, Gatterbauer *et al*[113] used an ELISA assay to determine the antibody types [IgM, IgG, IgA, and IgG subclass anti-GM1, anti-GQ1b, and anti-asialo-GM1 (anti-GA1)] that were present in patients presenting with neurological or other complicated neurological diseases. Increased anti-GM1 and/or anti-GA1 was found to be more frequent in lupus patients with central nervous system involvement than without[113]. Additionally, in 1999, a case report was published that found antibodies against ganglioside GM1 (indistinguishable from GBS) in the serum of a patient with SLE and a "drop foot", though no antibodies against *C. jejuni* were observed[114]. It has also been reported that an SLE like disease may be triggered in a Balb/c mouse animal model, after immunization of mice with formaldehyde-treated *C. jejuni* and Freud's complete adjuvant[115,116]. However, although *Campylobacter* species may be isolated from lupus patients, it is currently debatable whether *C. jejuni* is the causative agent of lupus disease *per se*, or is simply able to maintain itself in lupus patients due to the immunosuppressive treatment they receive. Particularly interesting are the two articles in which the authors show that they were able to induce an SLE like illness in Balb/c mice using formaldehyde fixed *C. jejuni*[115,116].

CELIAC DISEASE

Celiac disease is an autoimmune disease in which individuals possess antibodies against gluten protein, a protein found in wheat, barley and rye. Sufferers from celiac disease should avoid eating gluten-containing foodstuffs and therefore are subject to dietary restrictions. At least one report has indicated a role for *C. jejuni* in the aetiology of celiac's disease[117]. Additionally, a case report was published in 2010 of a girl suffering from celiac disease that was associated with recurrent Guillain-Barré syndrome (*C. jejuni* being one of the main microorganisms proven to be associated with Guillain-Barré syndrome)[118]. Alaedini *et al*[119] showed increased levels of ganglioside antibodies in celiac disease patients, and suggested that a pre-disposition of celiac patients to bacteria possessing cross-reactive lipopolysaccharides (LPS) such as *C. jejuni* (and *Haemophilus influenzae*), may predispose to the development of anti-ganglioside antibodies (similar to the aetiology of Guillain-Barré syndrome). A similar hypothesis involving tissue atrophy and degeneration of mucosa was also proposed by Sabayan *et al*[120] in 2007.

CARDIOMYOPATHY/MYOCARDITIS

Cardiomyopathy is a measurable deterioration of the function of the heart muscle, usually leading to heart failure. Common symptoms include breathlessness and peripheral oedema (*e.g.*, swelling of the legs). People

with cardiomyopathy are often at risk of dangerous forms of irregular heart beat and sudden cardiac death. The most common form of cardiomyopathy is dilated cardiomyopathy. Myocarditis is an inflammation of the myocardium (heart muscle) and is synonymous with the term inflammatory cardiomyopathy. Interestingly, *C. jejuni* has been linked to cardiac disease in several case reports[121-136]. Also, more severe cases of *C. jejuni* infection may result in heart failure of the patient[123,128,131]. In 2007, Becker *et al*[137] investigated whether the incidence of perimyocarditis is increased following *C. jejuni* infection. Their conclusion after screening 6204 patients for perimyocarditis, and after the patients had experienced a *C. jejuni* infection, was that the incidence rate of myocarditis was 16.1 (95%CI: 2.3-114.4) per 100000 person-year in the *Campylobacter* population compared to 1.6 (95%CI: 0.2-11.4) per 100000 person-year in the control cohort[137]. Although this observation was not found to be statistically significant, the authors did conclude that, based on the rarity of this condition and case reports in the literature linking *Campylobacter* cases with perimyocarditis, it could not be ruled out that a potential association between *Campylobacter* and perimyocarditis might exist[137]. Additional research, indicates that there seems to be a tendency for males to be overrepresented in cardiomyopathy patient groups following *C. jejuni* gastroenteritis symptoms[121-126,138-140], which warrants further investigation. Alzand *et al*[125], suggested that the mechanism by which *Campylobacter* causes myo(peri)carditis could be attributed to direct bacterial invasion of cardiac tissue, bacterial toxins, circulating immune complexes, or cytotoxic T-cells. However, at the moment, the mechanisms leading to cardiac disease after *C. jejuni* infection remain unknown, but support the idea that *C. jejuni* is able to cause systemic infections[9,10].

At this point in the review, the authors present evidence for an association between infection with *Campylobacter* spp. and (chronic) inflammatory diseases, which is based mainly on case reports in the scientific literature.

ACUTE TRANSVERSE MYELITIS

Acute transverse myelitis is a neurological disorder that affects the spinal cord through inflammation, generating for example complications such as axonal demyelination. The disease is associated with an infection or vaccination[141]. In two relatively recent case reports, from 2007 and 2012, acute transverse myelitis was associated with *C. jejuni*-induced gastroenteritis[141,142]. Patients were found to harbor cross-reactive antibodies against the sialylated LOS structures of *C. jejuni*, specifically high titres of anti-GM1 were observed.

GLOMERULONEPHRITIS

Glomerulonephritis is a renal disease that is characterized by inflammation of the glomeruli, or small blood vessels in the kidney[143,144]. It may present with isolated hematuria and/or proteinuria (blood or protein in the urine); or as a nephrotic syndrome, a nephritic syndrome, acute renal failure, or chronic renal failure[144]. Diagnosing the pattern of glomerulonephritis is important because the outcome and treatment differs in different types of glomerular disease[144]. The primary causes of glomerular disease are intrinsic to the kidney[144], but secondary causes of disease may be associated with; certain infections (bacterial, viral or parasitic pathogens), drugs, systemic disorders (SLE, vasculitis), or diabetes[143]. Several case reports have shown a potential link between *C. jejuni* infection and glomerular disease[145-150]. In some reports a *C. jejuni* antigen was identified in the glomeruli suggesting a causal role for this bacterium in the disease process[145,148].

VASCULITIS

Vasculitis is a group of autoimmune diseases in which blood vessels are attacked by the immune system and where inflammation is present[151]. *C. fetus* subsp. *intestinalis* was one the first *Campylobacter* species linked to vasculitis and is seen most often in older, debilitated, or chronically ill men[152]. In case reports, *C. jejuni* has been linked to patients experiencing various forms of vasculitis[114,146,153-158], though whether an actual causal relationship exists between disease and infection is as yet is unknown.

PSORIATIC ARTHRITIS

Psoriatic arthritis is a form of inflammatory arthritis that will develop in up to 30% of people who have the chronic skin condition psoriasis[159]. Psoriatic arthritis is said to be a seronegative spondyloarthropathy and therefore occurs more commonly in patients with tissue type HLA-B27[159]. A strong link between anti-*Campylobacter fetus* antibodies in psoriatic arthritis patients (rheumatoid arthritis, non-arthritic-psoriasis and psoriatic arthritis patients) was observed in the study of Lapadula *et al*[160]. Currently, no further studies on this subject have been reported, and it should be noted that the patient group used in the Lapadula study was small.

CANCER

C. jejuni is phylogenetically closely related to *Helicobacter pylori*, a bacterium established to be a causative agent of gastric cancer[161]. Further, the cytolethal distending toxin of *C. jejuni* may possess DNAse activity and could induce the breakage of double stranded DNA[162], one of the possible steps on the development of cancer. Currently, there is some evidence indicating that *C. jejuni* may possibly be linked to the development of mucosa-associated lymphoid tissue (MALT) lymphoma[163-165]. MALT is a cancer type that originates from B cells in the marginal zone of the MALT, and is also called extranodal marginal zone B cell lymphoma. However, a large

cohort study of Scandinavian patients who had tested positive for *C. jejuni*, and were followed over time (≥ 10 years) showed no increased risk of developing malignancies following an infection by *C. jejuni*[166]. Interestingly, the authors did find a decrease in respiratory cancers following an infection by *C. jejuni*.

CONCLUSION

Campylobacter species are able to induce both gastrointestinal and systemic infections in humans and have been linked not only to acute disease, but also to a wide range of (chronic) inflammatory diseases. In this respect, the organism is particularly associated with the development of neurological diseases such as GBS, MFS, and with reactive arthritis, diseases that are facilitated by the development of cross-reactive antibodies to *Campylobacter* sialylated LOS carbohydrate structures. However, the true role of *Campylobacter*-induced molecular mimicry in other human inflammatory diseases remains to be determined, though this review indicates that the actual role of *Campylobacter* infections in human disease may be largely underestimated. Therefore, further research is required in order to accurately determine the importance of *Campylobacter* infection in a wide range of (chronic) inflammatory diseases of humans.

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MINIREVIEWS

Tuberculosis and hematopoietic stem cell transplant: Review of a difficult and often underestimated problem

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Abstract

Recipients of solid organ transplants (SOT) and stem cell transplants (SCT) constitute a group of patients at risk for tuberculosis (TB) development. The prevalence of active TB in patients undergoing SOT is higher than in patients undergoing SCT, probably due to the shorter period of immunosuppression in the latter. We reviewed the importance of SCT in individuals with hematological malignancies. Most TB cases occur in transplant patients by reactivation of latent infection after immunosuppression, most often within the first year after transplant, leading to graft loss and in some cases, death. Relevant variables to assess the risk of TB infection in a transplant recipient include the donor's and recipient's medical histories, imaging results, microbiology and tuberculin skin test (TST) and interferon-gamma release assays (IGRA). TST is routinely performed in the donor and recipient before transplantation. If TST is > 5 mm in the recipient or > 10 mm in the donor, it is necessary to exclude active TB (pulmonary and renal). Chemoprophylaxis is recommended in TST (+) recipients and in recipients with recent seroconversion, in donors with a history of untreated TB or in contact with an individual with active TB, if radiological images are suspicious and the IGRA is (+). The drug of choice is isoniazid. These topics are herewith reviewed.

Key words: Tuberculosis; Prophylaxis; Transplant; Solid organ transplantation; Hematopoietic stem cell transplantation

Core tip: This review highlights the importance of stem cells transplant (SCT) in individuals with cancer and hematological malignancies. However, the risk of acquiring tuberculosis (TB) in this way, has received little attention, especially in developing countries. SCT candidates should be screened for TB with a careful medical history and chart review to ascertain any history of prior TB exposure, since immunocompromised individuals are at higher risk of latent TB progression to active disease. Finally, we mention the importance of the immune response, particularly in allogeneic stem cell transplants, because infection by intracellular microorganisms such as Mycobacterium TB, could be inhibited by the process named cell reprogramming.

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INTRODUCTION

Stem cell transplant (SCT), also known as pluripotent

hematopoietic cell transplant, was previously referred to as bone marrow transplantation (BMT) since that was the source from which hematopoietic stem cells (HSC) were preferably obtained. This procedure has become an essential therapeutic tool in modern medical practice. As a results of increasing knowledge on SCT, several dogmas that for years hindered development in this area of medicine, have been set aside. It is now established that: (1) the successful collection of HSC does not require the destruction of the receptor's BM; (2) HSC create their own space in the receptor's marrow via graft-*vs*-host effects; (3) several tumors can be eradicated as a result of a graft-*vs* tumor effect; (4) allogeneic SCT (alloSCT) can be conducted on an out-patient basis[1]; (5) allotransplants can be performed in elderly or frail individuals[2]; (6) allogeneic SCT (alloSCT) can be done without red blood cell or platelet transfusions[3]; and (7) in Mexico and in other emerging countries, allotransplant costs can be significantly diminished. These changes have led to increased availability of SCT to a greater number of patients in Mexico and other emerging countries thus offering, in some cases, a real curative option to patients that until recently had no access to this modern therapeutic modality[4]. Transplant recipients constitute a group of patients at risk of developing tuberculosis (TB) and that face great diagnostic and therapeutic dilemmas; the disease's clinical presentation tends to be atypical and the sensitivity of available diagnostic techniques is low. Moreover, anti-TB drugs are highly toxic and frequently interact with anti-cancer agents, rendering disease management difficult. Most TB cases in patients that have undergone transplantation are due to reactivation of latent infections following immunosuppression[5]. We reviewed the relevance of hematopoietic stem cell transplantation (HSCT) in individuals with hematological malignancies and thus at risk of acquiring TB.

EPIDEMIOLOGY

In the past decades, TB prevention programs in developed countries have decreased its incidence; however, in emerging countries it is still high. In Mexico, the incidence of TB in the general population is 14.5/100000 inhabitants, with important regional differences[6]. Mycobacterial infection was uncommon after BMT in the past and, until recently, was considered to be a rare complication, receiving little attention. In North American studies, incidence rates vary between 0.6% and 1%; however, in countries where it is more endemic, its incidence is higher: 1.6% in Spain, 5% in Hong Kong and in Taiwan[7].

A review of BMT patients in large US centers revealed an incidence rate of 0.49%-1%[8] and the scant data available in countries with a high incidence of TB referred frequencies ranging from < 1% to 5.5%[9] and reaching 16% in Pakistan, according to recent reports[10].

However, since the onset of the AIDS epidemic and the emergence of multidrug-resistant strains of *Mycobacterium tuberculosis* (MTB), there has been an increasing number of reports of mycobacterial infection in SCT recipients[11]. The lack of a significant response after corticosteroids and the initially predominant involvement of the upper lobes should raise the possibility of pulmonary tuberculosis. A high index of suspicion is important in establishing the diagnosis, and prompt and appropriate treatment will invariably improve the disease's outcome[12]. The reported frequency of MTB infection in solid organ transplant recipients varies from 0.2% to15% (mean, 3.7%), which is 6 to 62 times higher than its frequency in the general population (0.01%-0.045%)[13]. The incidence of TB in the general population is the principal predictor of the increased frequency observed in transplant recipients.

STEM CELL TRANSPLANTATION

SCT is a life-sustaining treatment indicated in some individuals with cancer and hematological malignancies[14]. HSCT refers to the infusion of hematopoietic stem cells obtained from a donor into a patient that has been treated with chemotherapy, usually myeloablative. HSCTs are classified as either allogeneic or autologous, depending on the source of the transplanted hematopoietic progenitor cells. HSCT is defined as any transplantation of blood or marrow-derived hematopoietic stem cells, regardless of the type of transplant (allogeneic or autologous) or cell source (bone marrow, peripheral blood, or placental/umbilical cord blood)[15].

The number of transplants performed in the United States has gradually increased over the last 20 years, particularly in older patients (50 years old). According to the Center for International Blood and Marrow Transplant Research summary report, there were 7012 allogeneic and 9778 autologous transplants performed in 2009[16].

SCT provides an increased chance of survival to patients facing hematological and other potentially life-shortening diseases. These malignancies include acute lymphocytic leukemia, chronic lymphocytic leukemia, acute myelogenous leukemia, chronic myelogenous leukemia, aplastic anemia, Hodgkin's and non-Hodgkin's lymphomas, and multiple myeloma. SCT is an expensive treatment and there is wide variation in insurance company coverage, with companies often only paying part of the total expenses. Transplant expenses vary depending on the specifics and type of transplant. A study was conducted between 2000 and 2004 by Saito *et al*[17] at the Dana Farber Cancer Institute/Brigham Women's Hospital, in 376 patients receiving high-dose SCT to estimate costs. The researchers reported median costs of up to $102574 and 36 d of initial hospitalization in a complicated allogeneic SCT.

Hematological disease is frequently accompanied by liver dysfunction. The principal causes of liver injury relating to SCT include: (1) high-dose cytoreductive therapy (chemotherapy and/or radiation) administered prior to transplantation and which may result in veno-occlusive disease (VOD) or nodular regenerative hyperplasia

Table 1 *Mycobacterium Tuberculosis* infections after allogeneic stem cell transplantation

Ref.	Country (period of study)	Patients with TB/No. of HSCT	TB incidence in general population (%)	Site of infection (*n*)
Navari *et al*[20]	United States (1983)	2/682	0.014-0.03	Lung
Kurzrock *et al*[21]	United States (1984)	2/90	0.014-0.03	Lung
Roy *et al*[8]	United States (1974-1994)	11/2241	0.014-0.03	Lung (1), EP (11)
Ip *et al*[22]	Hong Kong (1991-1994)	10/183	5.5	Lung
Aljurf *et al*[23]	Saudi Arabia (1986-1997)	4/641	0.62	Lung, CNS, spine
Budak-Alpdogan *et al*[19]	Turkey (1988-1998)	5/351	1.42	Lung (4), renal (1)
de la Cámara *et al*[11]	Spain (2000)	12/2866	0.41	Lung
Ullah *et al*[24]	Pakistan (2001-2006)	4/154	2.6	Lung (3), EP (1)
George *et al*[25]	India (1986-2001)	9/304	2.3	Lung (2), EP (7)
Lee *et al*[26]	South Korea (1996-2003)	9/295	3.1	Lung (8), EP (1)
Ullah *et al*[27]	Pakistan (2002-2007)	2/37	5.4	Lung
Shima *et al*[28]	Japan (2009)	Case report	-	EP

TB: Tuberculosis; HSCT: Hematopoietic stem cell transplantation; EP: Extrapulmonary; CNS: Central nervous system.

(NRH); (2) liver toxicity due to other drugs used after transplantation; (3) viral and bacterial infections; and (4) acute and chronic graft *vs* host disease (GVHD) in the case of allogeneic transplantation. The differential diagnosis of these complications is guided by knowledge of the timing of their appearance. NRH of the liver is a rare disorder characterized by diffuse micronodular transformation of the hepatic parenchyma, with areas of regenerative activity alternating with areas of atrophy and no fibrous septa between the nodules. Its presentation may be similar to VOD although it is associated with non-cirrhotic portal hypertension and ascites developing after day 100 post-BMT[18].

TB IN ALLOGENEIC STEM CELL TRANSPLANT RECIPIENTS

In general, TB is rarely seen in alloSCT recipients, but this observation has been challenged in developing countries such as Turkey, where TB infection is more prevalent than in Europe and the United States[19]. In this retrospective study, the incidence of TB infections in 351 alloSCT recipients was reported. The frequency of TB in alloSCT recipients after the allograft (5 of 351) was far greater than that in the general population (35.4 per 100000). Among the 351 patients who underwent alloSCT, 77 subjects that received isoniazid (INH) chemoprophylaxis for 6 mo did not develop post-transplant TB. However, 5 of the remaining 274 patients who received no chemoprophylaxis developed TB a median of 12 mo (range, 10-47 mo) after the allograft (Table 1).

In the bone marrow transplant population, despite severe immune suppression, there is a low incidence of mycobacterial infections[29] that contrasts with the experience reported in other immunosuppressed patients (AIDS and renal transplant recipients). This may be due, at least partly, to the more prolonged duration of immunosuppression in AIDS patients and in recipients of solid organ transplants, when compared with the usual BMT patient[30]. Most patients who develop TB after SCT do not have clearly identified risk factors[31]. Most had normal pre-transplant chest radiographs and no direct history of contact with TB. Although most cases of TB have occurred in alloSCT recipients, 20% have developed in autologous recipients. Despite this low rate[32], diagnostic vigilance must be maintained.

TB among transplant recipients may result from reactivation of quiescent M.tb foci, transmission by the graft or contamination by actively infected individuals. Graft transmission has been documented in renal, lung and hepatic transplants, but accounts for less than 5% of all TB cases in transplant recipients. The risk of TB development in transplant recipients is estimated to be 20 to 50 times higher than in the general population even in developed countries, and mortality rates vary between 20% and 40%. Risk factors include pulmonary images suggesting previous TB infection, immunosuppressive treatment with OKT3 or anti-T cell antibodies, diabetes mellitus, chronic liver disease and coexisting infections[33]. In patients undergoing SCT, associated risk factors include chronic GVHD, allogeneic transplant and total body irradiation[22].

Although accurate diagnosis may be difficult, it is currently possible to hypothesize and/or identify a fungal etiology of pneumonia in SCT recipients; however other pathogens such as *Mycoplasma pneumoniae* or MTB may present clinical and radiological pictures resembling mycosis in SCT patients[34].

PATHOGENESIS OF TB IN HSCT RECIPIENTS

TB is transmitted from person to person by respiratory droplets. Although some people develop active TB disease after infection, almost all TB infections are asymptomatic and remain latent. LTB progresses to active disease in approximately 5%-10% of infected individuals. The rate of progression is much greater in HSCT recipients. The risk of TB in transplant recipients is estimated to be 20 to 50 times higher than in general population even in developed countries, and mortality rates vary from 20% to 40%. Risk factors include pulmonary images suggesting previous TB infection, immunosuppressive treatment with OKT3 or anti-T cell antibodies, Diabetes mellitus, chronic liver disease and coexisting infections (Figure 1).

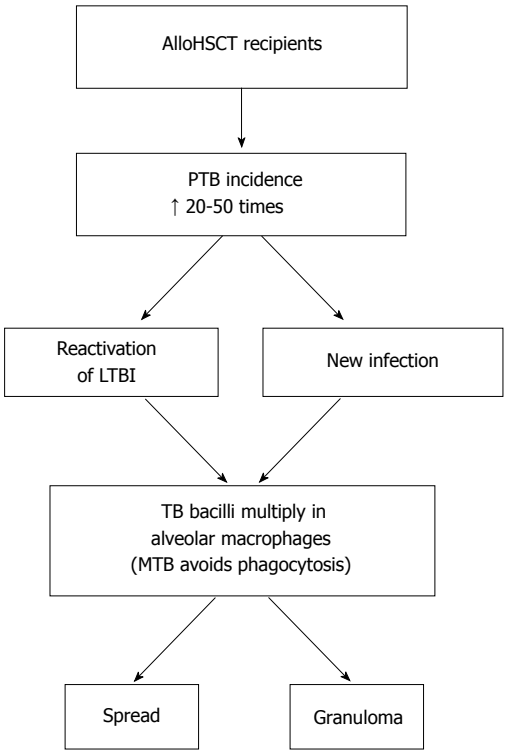

Figure 1 Pathogenesis of tuberculosis in hematopoietic stem cell transplant recipients. AlloHSCT: Allogeneic hematopoietic stem cell transplant; TB: Tuberculosis; PTB: Pulmonary TB; LTBI: Latent TB infection; MTB: *Mycobacterium tuberculosis*.

EVALUATION OF PATIENTS BEFORE SCT

SCT candidates should be screened for TB with a careful medical history and chart review to ascertain any history of prior TB exposure, since immunocompromised individuals have a higher risk of progression of latent TB (LTB) infection to active disease. Also, physicians should apply a tuberculin skin test (TST) using the Mantoux method with five tuberculin units of purified protein derivative or conduct an interferon-gamma release assays (IGRA) The sensitivity and specificity of IGRA testing methods varies according to the used kit type and the study population, and fluctuates between 50% and 100% and 85% and 100% respectively, in different studies (Table 2). Experts disagree on the convenience or benefit of routinely obtaining a TST or IGRA in every transplant candidate. Interpretation of the TST may also be complicated by a history of prior Bacillus Calmette-Guérin (BCG) vaccination, although tuberculin reactivity following BCG tends to wane over time[35]. Any patient with a recent positive TST or IGRA or a history of a positive test and no prior preventive therapy, should be evaluated for active TB. At a minimum, the patient should be asked about symptoms of systemic disease and respiratory symptoms such as cough and shortness of breath, and a chest radiograph should be assessed[36]. Any MTB-mediated disease either in the donor or recipient must be treated until complete microbiological and radiological resolution before considering the possibility of a transplant[37].

Table 2 Salient aspects for diagnosing tuberculosis in a hematopoietic stem cell transplantation recipient

Test	Sensitivity (%)[1]	Specificity (%)[1]	Indicates
TB skin test	72	35	LTBI or active TB
Acid-fast bacillus	50-80	98	Infection
Nucleic acid amplification test	80-98	95-99	Infection
Culture	70-90	98	Active TB
Serology	20-70	47-81	Active TB or infection
Interferon gamma release assays	50-99	85-99	LTBI or active TB
Chest radiography	47-73	76	Probable active TB

[1]These numbers are influenced by the epidemiological situation. LTBI: Latent tuberculosis infection; TB: Tuberculosis.

SCT center personnel should follow guidelines regarding the control of TB in healthcare facilities, including instituting airborne precautions and negative-pressure rooms for patients with suspected or confirmed pulmonary or laryngeal TB. Health care workers should wear N95 respirators, even in isolation rooms, to protect themselves from possible TB transmission from patients with active pulmonary or laryngeal TB, particularly during cough-inducing procedures[38]. SCT candidates and recipients should avoid exposure to persons or environments where there is a substantial risk of respiratory contact with individuals with active TB. It is prudent to advise SCT candidates and recipients that certain occupations (*i.e.*, volunteer work or employment in health care facilities, correctional institutions or homeless shelters) can increase their risk of TB exposure[39].

In SCT patients, a high incidence of TB might be expected due to the complex and severe immunodeficiencies that these patients undergo. Spain has a high incidence of TB (40-45 cases/10^5 inhabitants/year) and a high prevalence of infection (25%-29%) that increases to 56% in individuals > 49 years of age[40], the highest incidence of tuberculosis in Europe after Portugal[41]. It also boasts one of the highest transplant activity in Europe[42]. In a survey of TB after SCT, 20 confirmed cases were found (8 in autologous and 12 in allogeneic transplants) among 8013 patients. TB post-SCT was a late infection (172-324 d), most frequently limited to the lungs (80%) and less frequently, extrapulmonary or disseminated. All SCT patients with TB were symptomatic, fever and cough being the most common symptoms. In allogeneic transplant patients, TB was associated with a high mortality: 25%[11].

INDICATIONS FOR TREATMENT OF LATENT TB INFECTION (LTBI) OR PROPHYLAXIS

Because of the high risk of reactivation or the development of a new infection, prophylaxis should be ad-

Table 3 Clinical manifestations of nontuberculous mycobacterial disease in recipients of hematopoietic stem cell transplant and solid organ transplants

Transplantation type	Mycobacterium species	Types of infection
HSCT	MAC, *M. haemophilum, M. fortuitum, M. Chelonae, M. abscessus*	Catheter-related, pulmonary, cutaneous, disseminated
Kidney	*M. chelonae, M. kansasii, M. haemophilum, M.fortuitum*	Local cutaneous, disseminated, disseminated cutaneous, osteoarticular, pleuro-pulmonary
Heart	*M. kansasii,* MAC, *M. haemophilum, M. scrofulaceum*	Pleuro-pulmonary, disseminated, disseminated cutaneous
Lung	MAC, *M. abscessus, M. haemophilum, M. fortuitum*	Pleuro-pulmonary, local cutaneous, disseminated

HSCT: Hematopoietic stem cell transplant; MAC: *Mycobacterium avium*- intracellulare complex.

ministered to immunocompromised SCT recipients or candidates who: (1) Have been exposed to someone with active, infectious (*i.e.*, sputum-smear positive) pulmonary or laryngeal TB, regardless of the SCT recipient's or candidate's TST or IGRA status; (2) Have a positive TST result-regardless of prior BCG vaccination-without previous treatment and no evidence of active TB disease. A positive TST with a history of BCG vaccination is still considered by the American Thoracic Society as an indication for prophylaxis in patients who "have medical conditions that increase the risk for disease"[36], and which presumably include SCT; and (3) Have a positive IGRA result, without previous treatment and no evidence of active TB.

The report of a high frequency of reactivation of previously treated TB following transplantation, especially in some parts of the world where the endemic TB prevalence is high, suggests that these patients may be at high risk, and therefore, isoniazid (INH) prophylaxis should be considered[26]. LTBI therapy may carry a variable toxicity risk, particularly in the liver and requires strict plasma measurements of immunosuppressive therapy levels. To date, isoniazid is the drug of choice in prophylaxis and has proven effective. The value of prophylaxis in countries with a high rate of LTBI, or in SCT patients from such countries, should be considered at an institutional level.

INH is well tolerated after SCT even with concurrent fluconazole use[43]. Concurrent use of itraconazole is not recommended, and the impact of voriconazole or posaconazole is unknown.

BCG vaccination is contraindicated in SCT candidates. Disseminated BCG infection has been reported among immunocompromised individuals exposed to BCG[44].

Donors who live in or originate from countries where TB is endemic, are at an increased risk of developing TB or LTBI at rates similar to those in their population of origin. There is no known risk in transplanting hematopoietic progenitor cells from an untreated donor with latent or active TB[45].

NONTUBERCULOUS MYCOBACTERIAL INFECTION IN SCT RECIPIENTS

Nontuberculous mycobacteria (NTM) are ubiquitous environmental microorganisms that have generally been considered an uncommon cause of human disease. Before the AIDS epidemic, most cases presented as indolent, cavitating pulmonary infections in patients with other underlying lung diseases, such as chronic obstructive pulmonary disease or previous TB[46]. Mycobacterial infections after transplant have increased in frequency and severity, reflecting both increased exposure and improved diagnostic methods. In countries where TB is endemic, infections due to MTB are more frequent than are infections due to NTM[47].

NTM infections in HSCT recipients have been reported with an incidence ranging between 0.4% and 4.9%[48]. The clinical manifestations of NTM disease in HSCT and solid organ transplant (SOT) recipients are shown in Table 3. The clinical manifestations of disease in HSCT recipients differed from those in SOT recipients. The most common manifestations of NTM disease in HSCT recipients are central venous catheter-related infection, including exit site-related, tunnel-related, and catheter-related blood stream infections. Pulmonary and cutaneous disease is also commonly reported[49].

The most frequently isolated species in HSCT recipients are *mycobacterium avium*-intracellulare complex (MAC) and *M. haemophilum*. Rapidly growing mycobacteria, such as *M. fortuitum* is also common. MAC infection is most often associated with pulmonary or disseminated disease. Rapidly growing isolates have been predominately obtained in catheter-related infections. The presence of *M. haemophilum* has been reported more frequently in SCT recipients than in SOT recipients, usually in association with pulmonary or cutaneous disease but also manifesting as disseminated, osteoarticular, and catheter-related disease.

NTM disease has been reported in recipients of kidney[50], heart[51] and lung transplants[52]. Rapidly growing mycobacteria have been associated with disease in SOT recipients less often than in HSCT recipients.

RELEVANT IMMUNOLOGICAL ASPECTS OF STEM CELLS

Patients receiving transplanted hematopoietic cells undergo a period of immune dysfunction that lasts approximately a year and compromises both cellular and humoral immune mechanisms. This leads to a proclivity to develop infections in the post-transplant period. The

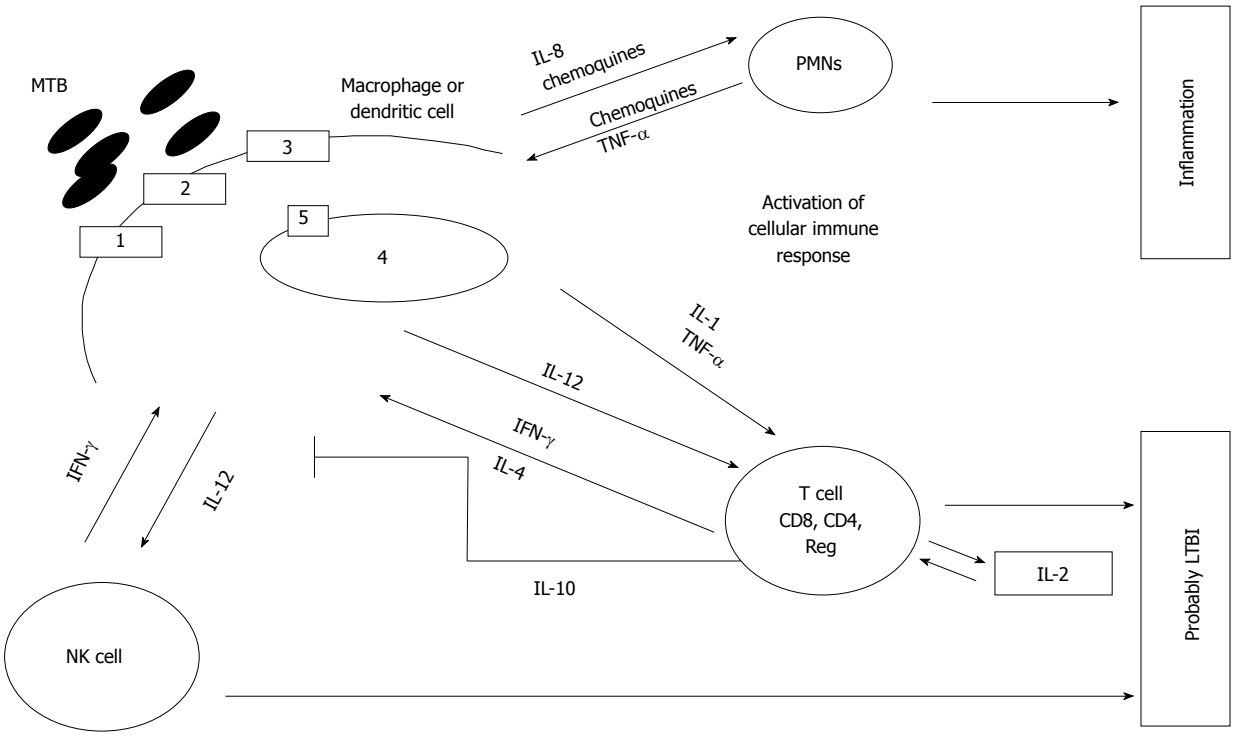

Figure 2 Cellular immune response against tuberculosis. *Mycobacterium tuberculosis* (MTB) recognition by specific receptors and tool like receptors activited signaling pathways that lead to cell activation and cytokine production. The activated macrophages secrete cytokines and chemokines that activate macrophages, T cells and neutrophils, producing inflammation. The T cells and natural killer cells producting gamma interferon with other cytokines that induct activation of macrophages contributing to the elimination of MTB or Latent tuberculosis infection. 1: Complement recepter; 2: Mannose recepter; 3: Scavenger receptor; 4: Phagosome; 5: Toll like receptor. IL: Interleukin; LTBI: Latent tuberculosis infection; NK: Natural killer; TNF-α: Tumor necrosis factor alpha; IFN-γ: Interferon gamma.

conditioning regimen to which patients are subjected destroys normal hematopoiesis and damages different hematological lineages such as neutrophils, monocytes and macrophages as well as mucosal cells, leading to a loss of this barrier's integrity, particularly in the gastrointestinal tract where flora becomes potentially pathogenic.

Recovery of immune function depends on the type of transplant, since in allogeneic transplants the donor's lymphoid cells must learn to recognize an extraneous microenvironment in the absence of a totally functional thymus; this delays immune reconstitution. Another factor affecting the period of immune function recovery is the dosage of cytotoxic therapy in order to allow secondary lymphoid organs to become repopulated. This is applicable in the absence of chronic graft-*vs*-host disease[53].

We must underscore the fact that after hematopoietic stem cell transplant, there is also loss of immune memory in the recipient and the donor's acquired passive immunity unreliably confers long-term immunity[54].

Allogeneic and peripheral blood transplants are associated with a longer immune recovery period than syngeneic and autologous transplants. $CD3^+$ T lymphocytes recover a short time after transplant particularly when more CD34 cells are infused. There is also a prolonged $CD4/CD8$ ratio inversion due to a decrease in $CD4^+$ and an increase in suppressor $CD8^+$ cells[55].

The immunomodulatory capacity of mesenchymal stem cells (MSC), adult multipotent cells, may prevent allogenic rejection by fomenting the avoidance of antigens when interfering with dendritic cell and T lymphocyte function; they thus have a local immunosuppressive effect due to their ability to secrete cytokines. This effect is potentiated when the cells are exposed to an inflammatory milieu or high concentrations of interferon gamma[56,57]. MSC can also inhibit lymphocyte proliferation induced by alloantigens and mitogens such as phytohemagglutinin and concanavalin A or by activation with anti-CD3 and anti-CD28 antibodies. MSC may also inhibit the expression of molecules mediating antigen presentation and in co-culture with peripheral blood mononuclear cells, they increase the proportion of T lymphocyte sub-populations with a regulatory phenotype[58,59].

In hematopoietic stem cell transplants (HSCT), T lymphocyte function remains altered for approximately a year and in allogeneic transplants, the immune repertoire is dominated by donor-derived T cells, particularly effector memory cells[60]. Interleukin-2 (IL-2) production by T Lymphocytes is decreased in response to mitogen stimuli and delayed hypersensitivity reactions are absent and only recover in the absence of Graft-*vs*-Host disease. $CD8^+$ cell cytolytic activity is compromised as underscored by an ineffective response to Epstein-Barr virus[61], a mechanism that may be very similar to that of MTB infection that fundamentally depends on the cellular immune response (Figure 2).

The final phase of cellular immune response recovery hinges on the need for new T lymphocytes from donor pre-thymic precursors in the case of allogeneic transplant.

These cells are processed by the recipient's thymic tissue rendering them tolerant to the allo-environment. There are differences in this lymphocytic "education process" depending on the recipient's age. Children and young patients have a more functional thymus and therefore, an increased recovery in the numbers of T lymphocytes within the first two years after transplantation[62].

In contrast, natural killer cell recovery does not require a functional thymus and develops rapidly within the first weeks after transplant[53]. Although stem cell transplantation is an artificial maneuver, when performed it gears immune mechanisms to take advantage of the stem cells' pluripotent capacity and plasticity; these characteristics are further reflected in organ and tissue regeneration as well as in immune modulation, particularly in immune suppression. Stem cells, particularly MSC, have been shown to inhibit T and B lymphocyte proliferation *in vivo* and *in vitro*, to support the development of regulatory T lymphocytes, to decrease the lytic activity of natural cytotoxic, natural killer and cytotoxic T cells, and to inhibit the risk of infection particularly in the early post-transplant period.

This proclivity to infection particularly in allogeneic stem cell transplants, by intracellular microorganisms such as MTB, could be inhibited by a process named reprogramming in which cells in late differentiation stages reactivate the program of stem cells and recuperate their pluripotentiality.

Tissues can be regenerated by cellular reprogramming and become a treatment strategy for various degenerative disease entities. However, this topic is beyond the scope of this review and is only mentioned because the safety and efficiency of reprogramming methods may represent an alternative, since it imitates the mechanisms used by cells during development; for instance, in cell reprogramming without the introduction of nucleic acids, embryonic fibroblasts have been reprogrammed for the first time with the transduction of the recombinant proteins of transcription factors Oct4, Sox2, Klf-4 and c-myc. However, there are still numerous obstacles to overcome, such as the proteins' short half-lives that require repeated applications and are inherently inefficient[54]. Cellular reprogramming can also be conducted with non-autonomic signals whereby the stem cells destined to a particular organ (multipotent cells) are placed in a similar milieu to that of early embryonic development and are capable of self-reprogramming into a pluripotent state, like embryonic stem cells. Thus, cells from the three embryonic layers (ectoderm, mesoderm, endoderm) can be generated and reflect a state of trans-differentiation[63]. This form of reprogramming is closer to normal cellular ontogenesis mechanisms[64].

CONCLUSION

In summary, transplantation centers should maintain a high level of suspicion of mycobacterial infection during the first 4 mo after transplantation, when mortality due to mycobacterial infections is at its peak. Due to the large numbers of unmatched donors in transplantation programs in countries with high TB prevalences, constant vigilance is required for early detection of mycobacterial infection in SCT recipients. The fact that autologous SCT recipients are immunosuppressed even before transplant, should also be considered.

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MINIREVIEWS

Role of chemokines and cytokines in the neuropathogenesis of African trypanosomiasis

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Abstract

Trypanosoma brucei spp. cause human African trypanosomiasis (HAT) or sleeping sickness in humans and nagana in animals. The early stages of the disease have no specific symptoms; however, the late stage of the disease involves neurological signs of the disease, including disturbance of sleep patterns from which the disease derives the name sleeping sickness. During the late stage of African trypanosomiasis parasites, increased numbers of white blood cells and levels of cytokines and/or chemokines are found in the brain parenchyma and/or cerebrospinal fluid of animal models and HAT patients. In this mini review, contemporary findings on how chemokines and cytokines are thought to play an important role in the central nervous system invasion by the parasites, inflammation and the neuropathology of the disease are discussed. The levels of various cytokines and chemokines, such as interferon-gamma (IFN-γ), interleukin-1 beta (IL-1β), IL-6, IL-10, tumor necrosis factor-alpha (TNF-α), C-C motif chemokine 2 (CCL2), CCL3, C-X-C motif chemokine 8 (CXCL8, IL-8) and CXCL10, in the cerebrospinal fluid (CSF) of HAT patients correlate with the severity or stage of the disease. Thus, these molecules are possible candidates for differentiating between early and late stage HAT. The role of cytokines and chemokines in parasite invasion of the central nervous system is also being elucidated. IFN-γ, TNF-α and CXCL-10 are some of the cytokines and chemokines now known to facilitate parasite penetration of the brain parenchyma. Interestingly, they also constitute some of the candidate molecules with potential to differentiate between stage 1 and 2 of HAT. The increased levels of cytokines, such as IL-1β, IL-6, IFN-γ and TNF-α, as well as prostaglandins, during African trypanosomiasis might contribute to the neurological dysfunctions that occur during HAT.

Key words: African trypanosomiasis; Chemokine; Cytokine; Central nervous system; Brain parenchyma; Cerebrospinal fluid; Neuroinvasion; Neuroinflammation; Neurological disturbances

Core tip: Human African trypanosomiasis (HAT) or sleeping sickness, caused by Trypanosoma brucei spp., is staged into an early hemolymphatic stage and a late meningoencephalitic stage. During the late stage parasites, increased numbers/levels of white blood cells, cytokines and/or chemokines are found in the cerebrospinal fluid of patients. In this mini review, contemporary findings on how chemokines and cytokines, such as interferon-gamma (IFN-γ), TNF-α, C-X-C motif chemokine 8 (CXCL8) and CXCL10, are thought to play an important role in the central nervous system invasion by the parasites, inflammation and the neuropathology of the disease and what might be candidates to differentiate between early and late stage HAT are discussed.

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INTRODUCTION

Three morphologically identical subspecies of the hemoflagellate protozoan parasite *Trypanosoma brucei* (*T. b.*), *T. b. brucei*, *T. b. gambiense* and *T. b. rhodesiense*, cause African trypanosomiasis, the latter two species are human infective. The disease is endemic to sub-Saharan Africa and is transmitted through a bite of a tsetse fly (Glossina sp.) during a blood-meal. *T. b. gambiense*, which is found in foci in large areas of West and Central Africa, causes a chronic form of human African trypanosomiasis (HAT) that lasts for several months to years. On the other hand, *T. b. rhodesiense*, with a much more limited distribution, is found in East and Southeast Africa and causes an acute form of the disease that lasts for several weeks to months[1,2].

Clinically, HAT is divided in to two stages: an early hemolymphatic stage (stage 1) and a late encephalitic stage (stage 2)[1,3,4]. However, the demarcations between these two stages of the disease are not clear, more so for disease caused by *T. b. rhodesiense* where there is rapid transition from stage 1 to stage 2[3]. In the early stage of HAT, a chancre might develop at the site of inoculation, followed by involvement of blood and lymphatic systems, which presents with general signs and symptoms of infection, chronic intermittent fever, headache, lymphadenopathy, splenomegaly and pruritus. In the late stage of the disease, there are signs of nervous system involvement, which present as sleep disorders, *i.e.*, dysregulation of the circadian rhythm of the sleep-wake cycle and a fragmentation of the sleeping pattern, neurological symptoms, including confusion, tremor, fasciculations, general motor weakness, hemiparesis, akinesia or dyskinesia, sensory disturbances with diffuse hyperpathia, abnormal movements and speech disorders, and psychiatric symptoms. If untreated, the disease will lead to coma and death in most cases. The patients die in a state of cachexia and also because of opportunistic infections[4]. Clinical symptoms of HAT are of a non-specific nature; thus, its diagnosis is confirmed by finding trypanosomes in the blood and lymph nodes or in the cerebrospinal fluid (CSF) using microscopy, the latter during the late stage of HAT. The serological test card agglutination trypanosomiasis test is used to screen for *T. b. gambiense* infections. The World Health Organization criteria for diagnosing stage 2 HAT is the finding of trypanosomes and/or a white blood cell (WBC) count of > 5/μL in the CSF[1,4].

Differentiating between the two stages of the disease is imperative before treatment can be begun[1] because of the differences between the drugs used to treat early and late stages of HAT in terms of ability to cross the blood-brain barrier (BBB) and toxicity. The drugs which are used to treat the late stage of the disease, melarsoprol, eflornithine and the nifurtimox-eflornithine combination treatment, permeate the BBB better but are more toxic than the drugs used to treat the early stage of the disease, suramin and pentamidine[3,4].

In this mini review, the role of chemokines and cytokines in the invasion of the central nervous system (CNS) by the parasite and the ensuing inflammation and neuropathology which makes the disease intractable and fatal in most cases will be discussed. Cytokines are a large group of immunoregulatory molecules. They play an important role in the control and pathogenesis of infectious diseases. Chemokines are involved in recruitment and retention of immune cells during inflammation and infection.

Table 1 Cytokines and chemokines with increased expression in the brain parenchyma of rodents and cerebrospinal fluid of human patients, more during late than early stage African trypanosomiasis

Site	Cytokine/chemokine	Ref.
Chemokines		
Rodent brain parenchyma	CCL2[1], CCL4, CCL5, CCL7, CCL9, CCL12, CCL19, CCL28 CXCL1, CXCL5, CXCL9, CXCL10[1], CXCL12, CXCL13[1], CXCL14, CXCL16,	[5,19]
HAT patient CSF	CCL2[1], CCL3, CXCL8 (IL-8), CXCL10[1], CXCL13[1]	[5,7,8,20-25]
Cytokines		
Rodent brain parenchyma	IFN-γ[1], IL-1α, IL-1β[1], IL-6, IL-10[1], TGF-β, TNF-α[1]	[10,19,26-28,30]
HAT patient CSF	IFN-γ[1], IL-1β[1], IL-6[1], IL-10[1], TNF-α[1]	[5,7,8,20,22,32-34]

[1]Expressed in both late stage rodent brains and HAT patients' CSF. CCL: C-C motif chemokine; CSF: Cerebrospinal fluid; CXCL: C-X-C motif chemokine; HAT: Human African trypanosomiasis; IFN: Interferon; IL: Interleukin; TGF: Transforming growth factor; TNF: Tumor necrosis factor.

CHEMOKINE AND CYTOKINE EXPRESSION IN THE CNS

Trypanosome infection results in activation of the immune system and induction of expression of various cytokines and chemokines in both HAT patients and animal models of the disease[5-11]. However, eventually the infection results in immunosuppression[12-14]. The cytokines and chemokines that are induced, both in the periphery and the CNS, play an important role in the control of the parasites but they also contribute to the inflammation and immunosuppression which occurs during the disease[6-11,15-17].

Increased expression of chemokines in the CNS has been observed during African trypanosomiasis. The expression of the chemokines C-X-C motif chemokine (CXCL) 1, CXCL2 (macrophage inflammatory protein-2, MIP-2), CXCL5, CXCL9, CXCL10, CXCL12, CXCL13, CXCL14, CXCL16, C-C motif chemokine (CCL) 2 (MCP-1), CCL3 (MIP-1alpha), CCL4, CCL5 (RANTES), CCL7, CCL9, CCL12 and CCL28 was found to be up-regulated in the brains of rodents infected with *T. b. brucei*[5,9,18,19]. Some of these chemokines are expressed at higher levels during late than early stage African trypanosomiasis (Table 1). CXCL9 and CXCL10 were the most highly up-regulated cytokines in the brain at later stages when parasites had invaded the CNS, compared to early stages of the disease before CNS invasion. The

Table 2 Cytokines and chemokines involved in Trypanosoma brucei spp. neuroinvasion

Cytokine/Chemokine	Trypanosome levels in the brain parenchyma of transgenic mice compared to WT mice	Ref.
Chemokines		
CXCL10	CXCl10$^{-/-}$ and CXCR3$^{-/-}$ mice had less trypanosomes in the brain parenchyma compared with WT mice.	[5]
Cytokines		
IFN-α/β	IFN-α/βR$^{-/-}$ mice had slightly less trypanosomes in the brain parenchyma compared with WT mice.	[15]
IFN-γ	IFN-γ$^{-/-}$ and IFN-γR$^{-/-}$ had less trypanosomes in the brain parenchyma compared with WT mice. Trypanosomes accumulated in the perivascular compartment, confined between the endothelial and the parenchymal basement membranes, in certain areas of the brains of both transgenic mice	[10]
IL-12	IL-12P40$^{-/-}$ mice had less trypanosomes in the brain parenchyma compared with WT mice.	[10]
TNF-α	TNFR1$^{-/-}$ mice had less trypanosomes in the brain parenchyma compared with WT mice.	[15]

CXCL: C-X-C motif chemokine; IFN: Interferon; IL: Interleukin; TNF: Tumor necrosis factor; WT: Wild type.

increased expression of both chemokines was found to be dependent on interferon (IFN)-γ[5]. CXCL10 was found to be predominantly up-regulated in parenchymal astrocytes of hypothalamic regions, optic chiasm and optic tracts at later stages of the disease[5]. Of these chemokines, CCL2, CCL3, CXCL8 (IL-8), CXCL10 and CXCL13 have been found to be increased in the CSF of patients with late stage HAT infected with either *T. b. gambiense* or *T. b. rhodesiense* more than non-infected control patients or patients with early stage HAT (Table 1)[5,7,8,20-25].

Several studies have reported the increased expression of cytokines in the CNS during trypanosome infection. The cytokines IFN-α/β, IFN-γ, interleukin (IL)-1α, IL-1β, IL-4, IL-6, IL-10, IL-13, transforming growth factor (TGF)-β and tumor necrosis factor (TNF)-α were found increased in the brains of rodents infected with *T. b. brucei*[10,18,19,26-30]. Some of these cytokines are also expressed at higher levels during late than early stage African trypanosomiasis (Table 1). It has been suggested that astrocytes might be the source of some of these cytokines since the levels of these cytokines were found to correlate with astrocyte activation[19]. Lymphocytes are the major source of IFN-γ in the brains of *T. b. brucei* infected mice[10]. *T. b. brucei* CpG-DNA stimulates macrophages to increase the production of IL-12 and TNF-α[31]; thus, macrophages and possibly microglia might be some of the major producers of these cytokines in the brain during *T. b. brucei* infections. Of these cytokines, IFN-γ, IL-1β, IL-6, IL-10 and TNF-α have been found to be increased in the CSF of patients with late stage HAT infected with either *T. b. gambiense* or *T. b. rhodesiense* more than non-infected controls or patients with early stage HAT (Table 1)[5,7,8,20,22,32-34]. On the other hand, the level of TGF-β was decreased in the CSF of patients with late stage HAT infected with *T. b. rhodesiense* compared to patients with early stage HAT but was higher than a non-infected control, where it was not detected in the latter[34].

CYTOKINES, CHEMOKINES AND TRYPANOSOME BRAIN INVASION

Taking into consideration that the expression of various chemokines and cytokines in the CNS correlate with presence of trypanosomes in the brains of animal models of the disease and CSF of HAT patients, there is a possibility these molecules play a role in the recruitment, mobility and retention, and also in the control of the levels, of the parasites in the CNS. The role which some of these molecules play in trypanosome invasion of the brain have been studied using transgenic animal models (Table 2)[35].

Of these molecules, the role of IFN-γ in trypanosome invasion of the brain was the first to be studied using transgenic mice[10]. Mice deficient of IFN-γ or its receptor had higher parasites in the blood but had less parasites and lymphocytes in the brain parenchyma compared to wild type (WT) mice. In these transgenic mice, the parasites accumulated in the perivascular space between the endothelial and parenchymal basement membranes of the post-capillary venules[10], suggesting that IFN-γ or factors induced by it are important for parasite crossing of the parenchymal basement membrane. The source of the IFN-γ was most likely lymphocytes since the levels of IFN-γ did not increase in recombination activating gene deficient mice (lacking mature B and T lymphocytes) and parasite penetration into the brain parenchyma was reduced in these mice. Mice deficient of IL-12 have reduced IFN-γ levels[36] and also have less parasites penetrating the brain parenchyma[10].

IFN-γ induces the production of the chemokine CXCL10, also known as IFN-γ-induced protein 10 (IP-10). Mice deficient of IFN-γ have reduced expression of CXCL10 compared to WT mice during trypanosome infection[5]. Transgenic mice lacking CXCL10 or its receptor CXCR3 also showed reduced parasites penetrating the brain parenchyma, although they had similar parasites in the blood compared to WT mice[5]. CXCL10 deficient mice did not have accumulation of parasites in the perivascular space, suggesting that other IFN-γ-induced molecules instead of CXCL10 play a role in IFN-γ dependent passage of parasites across the parenchymal basement membrane.

The role of TNF-α in trypanosome invasion of the brain was also studied using transgenic mice[15]. Mice deficient of TNF-α receptor 1 had higher parasites in the blood but had less numbers of both parasites and T lymphocytes in the brain parenchyma compared to WT mice[15]. *T. b. brucei* infected mice deficient of TNFR1

Table 3 Selected cytokines and associated neurological and neuroendocrine features of African trypanosomiasis

Cytokine	Possible neurological and neuroendocrine features associated with	Ref.
IFN-γ	Sleep pattern disruptions, hyperalgesia/hyperesthesia and pain	[41,60]
IL1β	Hyperalgesia/ hyperesthesia and pain, neurodegeneration	[28,29,61]
IL-6	Hypopituitarism and endocrine dysfunctions, sleep pattern disruptions, hyperalgesia/ hyperesthesia and pain	[38]
TNF-α	Hypopituitarism and endocrine dysfunctions, sleep pattern disruptions, hyperalgesia/hyperesthesia and pain, neurodegeneration	[28,29,38,41]

IFN: Interferon; IL: Interleukin; TNF: Tumor necrosis factor.

had less adhesion molecules, vascular cell adhesion protein 1 and intercellular adhesion molecule 1 compared to WT mice, suggesting that the induction of adhesion molecules through TNF-α signalling might play a role in the TNF-α facilitated parasite and T cell invasion of the brain parenchyma. Mice deficient of IFN-α/βR had reduced numbers of T lymphocytes and parasites in the brain parenchyma compared to WT mice, but the magnitude was not as pronounced as mice deficient of IFN-γ, TNF-α or CXCL-10 signalling[15]. Mice lacking the receptors of two other cytokines, IL-1R and IL-18R, had similar parasites in the blood, as well as parasites and T lymphocytes in the brain parenchyma, as WT mice[15], suggesting that these cytokines do not play a significant role in parasite penetration in to the CNS.

CYTOKINES, CHEMOKINES AND NEUROPATHOLOGY DURING AFRICAN TRYPANOSOMIASIS

Chemokines such as CXCL10 play a role in the attraction, mobility and/or retention of inflammatory cells into the CNS during African trypanosomiasis[5] and thus contribute to the neuroinflammation and morbidity seen in the late stage of the disease. High levels of CCL2, CCL3, CXCL8 and CXCL10 in the CSF were found to be associated with the severity of the disease and neurological signs which are characteristic of late stage HAT[5,7].

High levels of cytokines, such as IL-1β, IL-6, IFN-γ and TNF-α, in the plasma or CSF have also been found to be associated with the severity of the disease and neurological signs which are characteristic of late stage HAT[7,8,22,36,37]. However, as well as neuroinflammation, the role of cytokines in causing brain dysfunctions which result in neuroendocrine dysfunctions, neurological symptoms and/or sleep disorders (Table 3) have also been studied. In HAT patients, high plasma concentrations of IL-6 and TNF-α have been found to correlate with hypopituitarism and endocrine dysfunctions[38]. Endocrine dysfunctions result in some of the signs and symptoms of HAT, such as impotence, amenorrhea, infertility and lethargy. Chronically elevated concentrations of IL-6 and/or TNF-α during HAT might have a direct inhibitory effect on the hypothalamus-pituitary-thyroid or adrenal axis, resulting in reduced thyroid hormone and cortisol secretion[38]. TNF-α inhibitors have been shown to restore the hypothalamic-pituitary-adrenal axis in other chronic inflammatory diseases, such rheumatoid arthritis[39].

IL-6, IFN-γ and TNF-α can alter synaptic functions and are implicated in causing sleep pattern disruptions[40-45]. IFN-γ alters clock gene expression and circadian rhythms in the suprachiasmatic nucleus (SCN)[41,46]. The SCN is essential for the generation and maintenance of daily rhythms in physiology and behavior[47-49]. IL-1β, TNF-α and IFN-γ also affect hypothalamic and brainstem neurons which are involved in sleep-wakefulness regulation[41,50,51]. Apart from HAT, IL-6 and/or TNF-α are elevated in other disorders associated with excessive daytime sleepiness, such as sleep apnea, narcolepsy and idiopathic hypersomnia[43,44,52].

Cytokines and chemokines can sensitize and stimulate nociceptors in the periphery and/or synaptic targets in the CNS, which can result in neuropathic pain[53]. Administration or up-regulation of IL-1β, IL-6, IFN-γ and TNF-α can induce neuropathic pain in rodents[40,54-59]. It has been suggested that IL-1 and IFN-γ might be implicated in the thermal hyperalgesia observed in *T. b. brucei infected* rats[60,61]. Hyperesthesia is one of the clinical features reported in HAT patients[3,4]. Thus, these cytokines, together with other inflammatory molecules, most likely contribute to the hyperalgesia/hyperesthesia and pain observed in HAT.

In rats infected with *T. b. brucei*, apoptosis of some cells and degeneration of some nerve fibres, although modest, in the brain have been found to be spatially associated with mRNA expression of the cytokines IL-1β and TNF-α[29]. Intraventricular infusion of an antagonist of TNF-α, but not IL-1, was found to reduce trypanosome-induced neurodegeneration[28]. Infusion of antagonists of both cytokines further reduced the trypanosome-induced neurodegeneration; thus implying that TNF-α is a principle mediator of trypanosome-induced neurodegeneration and its effects are augmented by IL-1[28].

CONCLUSION

The expression of cytokines and chemokines in the brain and/or CSF is increased in animal models of African trypanosomiasis and HAT patients and the levels of these molecules correlate with the severity or stage of the disease. The high levels of chemokines and cytokines in the brain and CSF during late compared to early stage African trypanosomiasis are most likely due to the invasion of the CNS by trypanosomes and/or WBCs in the late stage, resulting in neuroinflammation. Thus, these molecules are possible candidates for differentiating between early and late stage HAT. In the future,

clinicians could utilize this knowledge to treat patients with high levels of these molecules in the CSF as late stage patients; thus, possibly reducing the occurrence of relapses in late stage HAT patients who might have been wrongfully diagnosed as early stage and treated as such using the current staging criteria. Recently, extensive research has been undertaken to evaluate the suitability of these molecules as stage biomarkers and also as markers for treatment outcome in HAT patients[5,7,8,20-25,32-34]. The role of cytokines and chemokines in parasite invasion of the CNS is also being elucidated. IFN-γ, TNF-α and CXCL-10 are some of the cytokines and chemokines now known to have a facilitative role in parasite penetration of the brain parenchyma. Interestingly, they also constitute some of the molecules with potential to differentiate between stage 1 and stage 2 of HAT[5,20,22]. Moreover, neopterin, a stable product produced by IFN-γ activated immune cells, has been suggested to have potential to differentiate between these two stages of HAT[24]. The increased levels of cytokines, such as IL-1β, IL-6, IFN-γ and TNF-α, during African trypanosomiasis contribute to the neurological dysfunctions that occur during HAT. Thus, studying cytokines and chemokines during African trypanosomiasis not only aids in understanding the neurobiology of the disease, but also provides candidate diagnostic markers and possible therapeutic targets to reduce the neurological sequelae in surviving patients.

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CASE REPORT

Primary lymphocutaneous nocardiosis associated with gardening: A case series

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Abstract

Most cases of nocardiosis are seen in immunocompromised patients. Primary lymphocutaneous is a relatively uncommon presentation of this disease that may also occurs in normal hosts. Diagnosing this infection requires a high index of suspicion since cultures can take several days to exhibit growth. The microbiology laboratory must therefore be notified about cases in which this pathogen is suspected. We report four cases of primary lymphocutaneous norcardiosis. Of particular interest is the association of three of these cases with gardening.

Key words: Nocardia; Nocardiosis; Lymphocutaneous; Brasiliensis; Asteroides; Gardening

Core tip: Nocardiosis is an infection most often seen in immunocompromised individuals. In particular, primary cutaneous disease rarely occurs in normal hosts. We present a case series of patients that developed this infection after gardening. As nocardial infections are frequently mistaken for routine pyogenic processes and as routine cultures are rarely kept long enough to show growth, this condition should be considered in patients with such a history and cultures should be incubated for several weeks.

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INTRODUCTION

Nocardia species are ubiquitous soil-dwelling Gram-positive branching beaded bacteria that are responsible for a wide spectrum of diseases in both normal and immunocompromised patients[1,2]. Nocardia infections of the pulmonary system and disease dissemination, including secondary cutaneous involvement, are well documented in immunosuppressed patients[3,4]. We report four cases that demonstrate the classic features of primary lymphocutaneous nocardia infection. Three of these infections were associated with gardening.

CASE REPORT

Case 1

A 49-year-old Hispanic man with a history of diabetes mellitus presented with swelling and redness in the left elbow after squeezing a furuncle on his left hand 2 d prior. He had no fever and did not recall any injury or trauma to the hand. On physical exam, he had a furuncle with visible pus on the dorsum of the left ring finger. He also exhibited erythema and edema up to the left elbow with streaks of lymphangitis. He had a normal white blood cell count (WBC) count of 7.0 K/μL and an elevated high-sensitivity C-reactive protein (hs-CRP) of 6.6 mg/L.

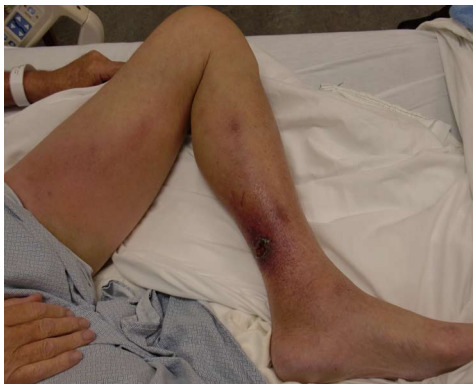

Figure 1 ***Nocardia brasiliensis*** **cutaneous abscess with lymphangitis.**

He was admitted and treated with intravenous clindamycin for 2 d. The lesion was incised and drained and the purulent fluid was sent for cultures. He was discharged on oral cephalexin and trimethoprim-sulfamethoxazole for suspected methicillin-resistant Staphylococcus aureus. The Gram stain revealed the presence of Gram-positive beaded rods 3 d after admission. *Nocardia brasiliensis* was found by sequence identification 25 d after the initial admission. Treatment with oral trimethoprim-sulfamethoxazole was continued for 6 mo.

Case 2

A 59-year-old Hispanic man with a history of esophageal cancer with metastases to the lung and hypertension presented to his primary care physician with left calf redness, swelling and pain. The symptoms started after manipulating a furuncle on his left calf. Two days prior to this, he had been working in his garden and had knelt down in soil. He had not received any chemotherapy in the last 2 years. He was started on oral cephalexin but after 2 d, he developed rigors, diaphoresis and fever. He therefore returned to the emergency room and was admitted. On physical exam, he was afebrile and had a 1 cm ulcer with purulent discharge on the posterior aspect of his left calf. The surrounding area was erythematous and tender but not fluctuant or indurated. He had an area of erythema on the medial aspect of the left thigh with tender left inguinal lymphadenopathy. The largest lymph node was about 1 cm in diameter. The patient exhibited lymphangitis along the medial aspect of the lower extremity (Figure 1). He had a normal WBC count of 8.2 K/μL, elevated CRP of 31.2 mg/L, alkaline phosphatase of 341 U/L, AST of 41 U/L and total bilirubin of 2.2 mg/dL. The wound was incised and drained and the fluid sent for cultures. He was started on vancomycin and piperacillin/tazobactam. The Gram stain showed Gram-positive beaded rods 3 d after admission. He was discharged on oral trimethoprim-sulfamethoxazole. Final cultures were positive for *Nocardia brasiliensis* by sequence identification 37 d after admission. Treatment with oral trimethoprim-sulfamethoxazole was continued for a total of 6 mo with complete resolution of symptoms.

Case 3

A 78-year-old Caucasian man with a history of prostate cancer and coronary artery disease presented to his primary care physician with a right knee lesion that had started as a small furuncle one week prior. He had been gardening and kneeling in the soil recently. Due to concerns for septic arthritis, an aspiration of the knee was attempted but no fluid was recovered. He was started on an outpatient regimen with oral doxycycline. After 2 d, he developed a new erythema over his right thigh. He therefore returned to the emergency room and was admitted to the hospital. On physical exam, he was afebrile and his right knee was erythematous. He had a suppurative lesion in the subpatellar region and there were multiple indurated and erythematous areas in a linear pattern from the patella to just below the femoral ligament. There was no inguinal lymphadenopathy. He had a normal WBC count of 8.6 K/μL and an elevated CRP of 56 mg/L. The area was incised and drained and the fluid sent for cultures. He was started on intravenous vancomycin. He was discharged after 2 d on oral trimethoprim-sulfamethoxazole. Fungal cultures were positive for partial acid-fast thin-branched filaments 6 d after admission. The organism was identified as *Nocardia brasiliensis* 39 d after admission. Trimethoprim-sulfamethoxazole was stopped because of a rising creatinine level and he was switched to amoxicillin/clavulanate and minocycline. The treatment was continued for a total of 6 mo with complete resolution of symptoms.

Case 4

A 62-year-old Caucasian man living in the Bahamas presented to his primary care physician with a right knee lesion associated with pain, redness, swelling and warmth for the last 3 d. About a week prior to developing these symptoms, he had noticed a small scratch in the same area. This wound had not been covered while gardening and he had been kneeling in soil on his bare knees. He had a past medical history of ulcerative colitis being treated with infliximab. As his symptoms worsened, he decided to seek care at our facility. On physical exam, he was afebrile, had a 2 cm × 3 cm × 1 cm abscess on the right knee, right inguinal lymphadenitis and signs of lymphangitis along the medial thigh. He had a normal WBC count of 7.0 K/μL and an elevated CRP of 24.5 mg/L. His alkaline phosphatase was 69 U/L, aspartate aminotransferase (AST) 67 U/L, and alanine aminotransferase 45 U/L. He was treated with intravenous ceftriaxone and his infliximab therapy was put on hold. Gram positive beaded rods were seen on the Gram stain 3 d after admission. The organism was identified as *Nocardia brasiliensis* 10 d later. He was then switched to oral trimethoprim-sulfamethoxazole. After 6 mo of therapy, his infliximab therapy was resumed. Given the need for treatment with his immunomodulator, he was continued on prophylactic trimethoprim-sulfamethoxazole indefinitely.

DISCUSSION

The Nocardia genus belongs to the order Actinomyceta-

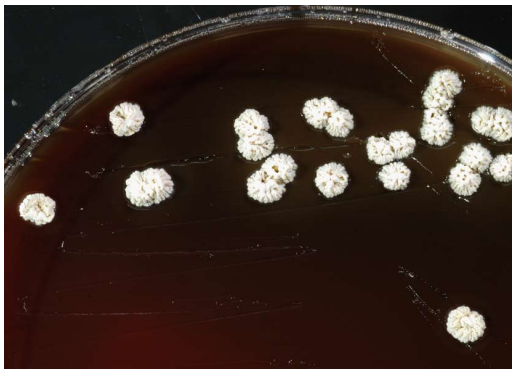

Figure 2 *Nocardia brasiliensis* white, rugged colonies on blood agar.

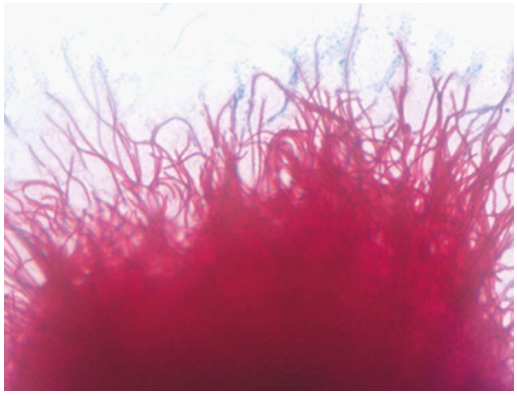

Figure 3 Modified acid fast stain of *Nocardia brasiliensis* demonstrating filamentous growth.

les, a group of gram-positive, aerobic, branching, beaded, partially acid-fast bacteria[5]. They are ubiquitous in nature and can be found in soil, air, and water[1,6]. *Nocardia asteroides* is the most common species associated with human disease[7]. There may be geographic variation in Nocardia species distribution with more case of *Nocardia brasiliensis* observed in the Southern United States[4].

The exact incidence of nocardiosis is difficult to estimate but approximately 1000 cases occur annually in the United States[8,9]. Nocardia infections of the pulmonary system and disease dissemination, including secondary cutaneous involvement, are well documented in immunosuppressed patients[1,2]. Primary cutaneous nocardiosis accounts for up to 5% of all cases of nocardiosis and is more often associated with *Nocardia brasiliensis*[2,3,10,11].

Primary cutaneous nocardiosis results from direct inoculation of the organism into the skin as a result of minor trauma, thorn puncture or insect bite[5,10,11]. Primary cutaneous nocardiosis may cause ulceration, pyoderma, cellulitis, nodules or subcutaneous abscesses. Patients commonly present with pain, swelling, erythema and warmth[2,3,6]. It can be clinically indistinguishable from other bacterial infections such as the ones caused by *Staphylococcus aureus* and group A streptococci.

Lymphocutaneous nocardiosis occurs when a primary nocardial skin infection involves and spreads within the lymphatic system. The clinical picture of lymphocutaneous nocardiosis could look similar to infections caused by *Sporothrix schenckii* or atypical mycobacterial infection[7,10]. However, in nocardia infections, the presentation is usually more acute.

Nocardia are relatively slow-growing organisms that can be grown on standard blood agar but prefer enriched media such as Lowenstein Jensen or Sabouraraud-dextrose agar. They typically appear as white, yellow, or orange rugous colonies (Figure 2). On microscopy, nocardia appears as aerial filaments that break up into small bead-like spores (Figure 3). Routine cultures usually require 5-21 d to exhibit growth, which may lead to underdiagnosis if the organism is not seen on Gram staining since most routine cultures are discarded after 3 d of incubation[6].

The standard treatment for nocardiosis is trimethoprim-sulfamethoxazole. Surgical debridement of purulent lesions may be needed to optimize therapy. While no definitive length of treatment has been established, the patient should be treated for at least 6 mo to prevent relapse. Longer treatment courses should be considered in immunosuppressed patients[2,6,8,10,12].

Our case series is of particular interest because three of the four patients had had recent exposure to soil while gardening. In addition, two of them recalled having a small wound on their knee prior to kneeling on soil. We believe that the patients inoculated themselves with the organism at that time. We therefore suggest that a careful history should be taken with specific questions about soil exposure in patients presenting with symptoms similar to those described. In addition, we suggest that a modified acid-fast stain be performed and that cultures be kept for 21 d in such cases.

Nocardiosis is an infection most often seen in immunocompromised individuals. Infections most commonly affect the lungs and are more often associated with *Nocardia asteroides*. Primary cutaneous disease rarely occurs in normal hosts and is typically associated with significant soil contact. In these cases, the main pathogen is *Nocardia brasiliensis*. Nocardial infections are frequently mistaken for routine pyogenic processes, as routine cultures are rarely kept long enough to show growth. Sulfa-containing regimens are often effective in treating nocardia infections and treatment should be continued for at least 6 mo.

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