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TITOLO TESI

Simkania negevensis: clinical and laboratory studies in the population of hemodialized and kidney transplanted patients.

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Index

Introduction

Chlamydias are obligate intracellular parasites of vertebrates, of some arthropod species, and of several free-living amoebae (1,2,3). They exhibit a peculiar two-stage developmental cycle that includes an extra- cellular infectious elementary body and an intracellular vegetative reticulate body. A further infective stage, the crescent body, was recently described for the *Parachlamydiaceae* (4), a new family within the order *Chlamydiales*.

The family *Chlamydiaceae* comprises two genera, *Chlamydia* and *Chlamydophila* (5). While *Chlamydia* species seem to infect only mammals such as humans, rodents, and swine, host specificity for *Chlamydophila* species is less strict, including for amphibians, reptiles, birds, and mammals (6). Both *Chlamydia* and *Chlamydophila* species comprise important human pathogens . *Chlamydia trachomatis* is a common cause of urogenital infection in humans and the agent of trachoma, one of the leading infectious causes of blindness worldwide. *Chlamydophila pneumoniae* is another important human pathogen, causing mainly respiratory infections. *C. pneumoniae* might also be involved in the pathogenesis of atherosclerotic cardiovascular diseases and neurodegenerative syndromes (7). The other species are mainly veterinary pathogens, though some of them may cause rare but severe anthropozoonotic infections, such as psittacosis due to *Chlamydophila psittaci* or zoonotic abortion due to *Chlamoydophila abortus* (8,9,10).

Historically, the term “chlamydia-like organisms” has been applied to refer to any intracellular microorganism that, like *Chlamydiaceae*, exhibited a two-stage developmental cycle. Since phylogenetic molecular analyses performed on some of these chlamydia-like organisms have showed this group to be polyphyletic, the term “chlamydia-like organisms” is only descriptive and retains no taxonomic value. Other authors (11) proposed the term “environmental chlamydiae” to refer to chlamydial organisms that fall outside the family *Chlamydiaceae*, with the latter being designated “pathogenic chlamydiae.” However, such a distinction seems inadequate, since a growing body of evidence supports the pathogenic role of some of these chlamydiae (12,13). By analogy to the term “amoeba-resistant microorganisms” (14), the term “amoeba-resisting chlamydias” could also be applied to the species that may infect and survive within amoebae, i.e., *Parachlamydiaceae*, *Simkania negevensis*, and *Waddlia chondrophila (15,16,17).* Indeed, most of these new species exhibited symbiotic or lytic interaction with amoebae. Nevertheless, since not all

new species of chlamydiae have been tested for their ability to resist destruction by free-living amoebae, the more general term “novel chlamydiae” should be preferred to designate all the chlamydiae not belonging to the *Chlamydiaceae*. These chlamydiae, which have recently been discovered and assigned to new families, are currently being investigated for their role as emerging pathogens.

Simkania negevensis (family *Simkaniaceae*) and *Parachlamydia acanthamoebae* (family *Parachlamydiaceae*) could represent important respiratory pathogens in humans, while *Waddlia chondrophila* (family *Waddliaceae*) seems to be a new agent of abortion in ruminants.

Molecular studies performed on human and animal samples have demonstrated the wide biodiversity and broad host range of chlamydiae. Thus, additional *Parachlamydiaceae* species, such as *Neochlamydia* sp., and a large variety of new 16S rRNA gene phylotypes have been detected in humans, cats, Australian marsupials, reptiles, and fishes , as well as in various environmental samples.

Moreover, new members of the *Chlamydiales* infecting invertebrates have recently been characterized. These include *Fritschea bemisiae* and *Fritschea eriococci* (family *Simkaniaceae*), which infect homopteran insects, and *Rhabdochlamydia porcellionis* (14) and *Rhabdochlamydia crassificans*, which infect the woodlouse *Porcellio scaber* (Crustacea: Isopoda) and the cockroach *Blatta orientalis* (Insecta: Blattodea), respectively. The presence of chlamydiae in arthropods is interesting, since arthropods were not previously considered to play a role in the epidemiology of chlamydial infections, with the only exception being flies as a vector of the agent of trachoma. To date, if we exclude cases of possible *Rhabdochlamydia*- related uveitis in humans, there is no hint that these invertebrate-associated chlamydiae may be pathogenic to humans.

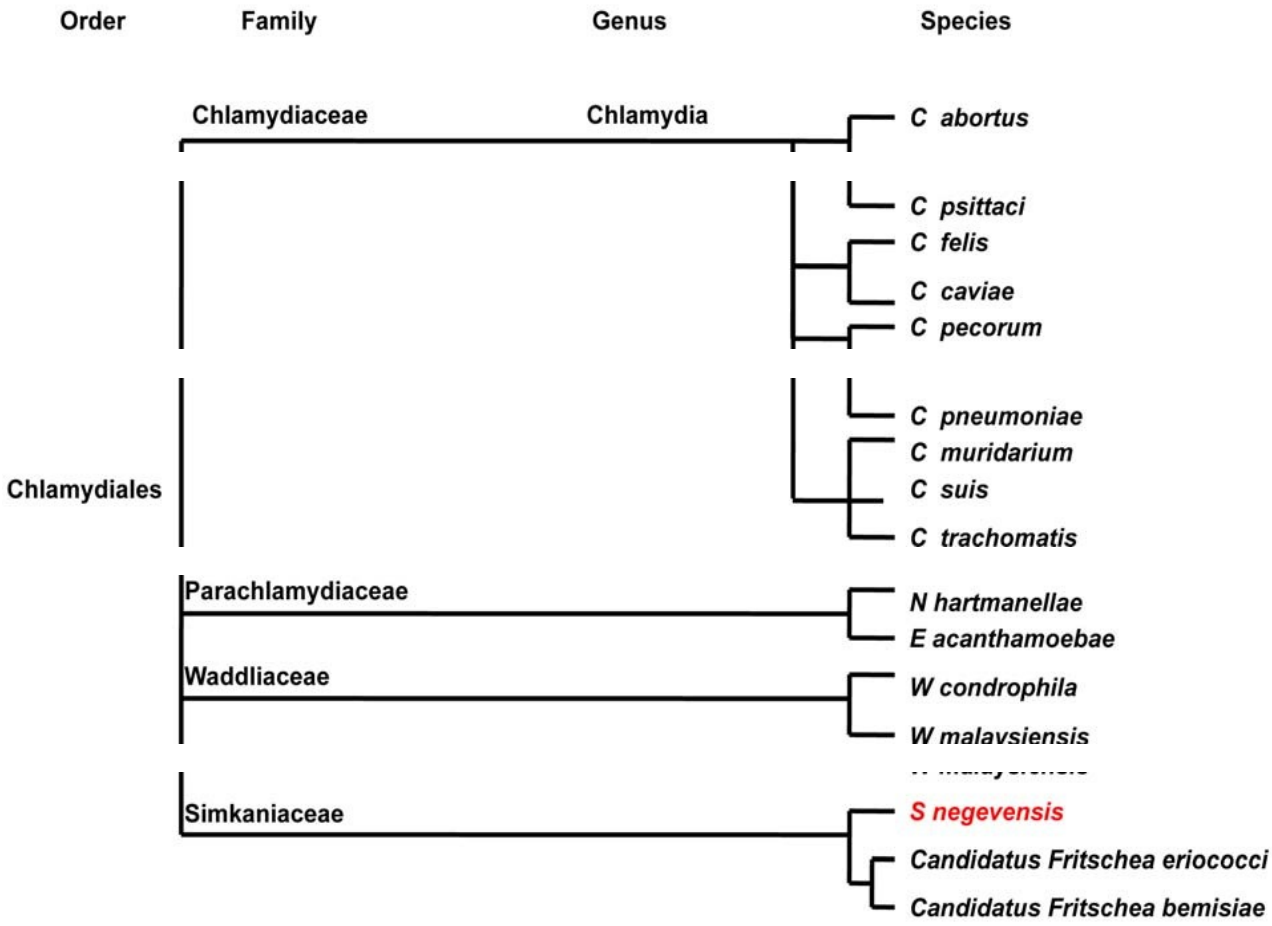
Evidence for a much larger biodiversity within *Chlamydiales* was afforded by several molecular studies performed on humans, animals, and environmental samples.

Simkania negevensis is an intracellular bacterium that was first detected when rapidly moving particles were observed in cytoplasmic inclusions in laboratory stocks of Vero cells. Trasnsmission electron microscopy of thin section s revealed small, dense forms and larger, homogenous forms, similar in appearance to chlamydial elementary and reticulate bodies.

For years in the order of Chlamydiales was this one family , that of Chlamydiaceae containing only one genus , Chlamydia , and nine species : Chlamydia trachomatis , Chlamydia pneumoniae ,

Chlamydia psittaci , Chlamydia pecorum , Chlamydia suis , felis Chlamydia , Chlamydia abortus , Chlamydia muridarum and Chlamydia caviae . The species C. trachomatis , C. pneumoniae and C. psittaci are responsible for human infections . C. trachomatis cause eye infections and urogenital , C. pneumoniae causes respiratory infections , while C. psittaci causes psittacosis - ornithosis (19) . Recently(1999) , the introduction of data of the phylogenetic analysis of the 16S and 23S ribosomal RNA in studies of taxonomy , brought significant alterations in the order Chlamydiales with the recognition of four separate groups of chlamydiae , framed in terms of " family " .

In the new classification, the family gathers Chlamydiaceae chlamydiae already known . Three other families , created to frame the new bacteria taxonomically " Chlamydia -like" recently discovered , are represented by the family Parachlamydiaceae with two species Parachlamydia acanthamoebae and Neochlamydia hartmannellae , family Waddliaceae with the only species Waddlia chondrophila and family Simkaniaceae with ' only species Simkania negevensis . These new families have more than 80% homology to the sequences of the 16S rRNA genes of Chlamydia (21).


Order
Family
Genus
Species
Chlamydiaceae
Chlamydia
C abortus
C psittaci
C felis
C caviae
C pecorum
Chlamydiales
C pneumoniae
C muridarium
C suis
C trachomatis
Parachlamydiaceae
N hartmanellae
E acanthamoebae
Waddliaceae
W condrophila
W malaysiensis
Simkaniaceae
S negevensis
Candidatus Fritschea eriococci
Candidatus Fritschea bemisiae

Bacterial cell

The bacteria belonging to the order Chlamydiales are particular microorganisms, similar to viruses for their parasitism obliged, and bacteria for mode of replication, antibiotic susceptibility, and the presence in them of DNA, RNA and ribosomes similar to those of the bacteria. Since metabolic point of view are able to synthesize autonomously various organic substances of low molecular weight.

The main metabolic defect, because of their intracellular parasitism obliged, is represented by the absence of any enzyme system for the production of energy (ATP), therefore, are forced to source of ATP and other cofactors (NAD, CoA, etc.) from host cell.

The bacterial structure is comparable to that of the Gram-negative bacteria, with a second membrane that, in the outer portion, has the characteristic component lipopolysaccharide (LPS or endotoxin) and by protein OMPs (Outer Membrane Proteins). The main protein is indicated by the acronym MOMP (Major Outer Membrane Protein), which has a structural function and metabolic as it acts as porin, allowing the entry of nutrients and essential molecules. Quite unique is the absence of peptidoglicanica component of the cell wall, replaced by a layer protein rich in cysteine (two proteins of 60 kDa and 15 kDa, respectively called large and small CRP or cystein-rich protein) that appears to be a functional equivalent of peptidoglycan, giving rigidity to the outer membrane through disulfide inter- and intra -molecular between proteins themselves.

To the particular structure of outer casings seems to be related the ability of these microorganisms to prevent fusing with lysosomes, the phagosome in which they are introduced inside the cell parasitized. The absence of a wall formed by peptidoglycan explains their low or no sensitivity to beta-lactam antibiotics (and in any case to drugs that work by preventing the synthesis of peptidoglycan).

The polypeptide pattern of S. negevensis obtained by SDS PAGE, is quite different from that of other members of the Chlamydiales. Only certain proteins, mainly heat shock, are shared by S. negevensis, W. chondrophila, P. acanthamoebae, C. pneumoniae and C. trachomatis.

The analysis by immunoblot of cell lysates of S. negevensis by murine hyperimmune serum shows the predominant membrane antigens, which are a set of three bands of molecular weight between

37-42 kDa, and additional bands of 26, 52 , 58, and 64 kDa, and a band of PM greater than 100 kDa.

Monoclonal antibodies (MAbs) prepared against whole organisms or against cell lysates, react primarily with the set of bands between 37 and 42 kDa, indicating strong immunogenicity of the complex. Some of these MAbs were able to neutralize in vitro S. negevensis, but unable to neutralize C. trachomatis in the same conditions. When these MAbs specific for S. negevensis were tested by immunoblot of cell lysates prepared or membrane of C. trachomatis, they were able to recognize an epitope localized on the protein OmpA. On the other hand, the MAbs that recognize epitopes family-specific OmcB (OMP-2), OmpA (MOMP), and does not bind LPS antigens of S. negevensis. The structure and function of the three polypeptides belonging to the complex outer membrane of S. negevensis needs further study. It is possible that the shared epitope constitute part of a porin common to Chlamydiaceae and S. negevensis (21,22).

Taxonomic relationships

S negevensis belongs to a new family, Simkaniaceae, in the order Chlamydiales based on its obligately intracellular parasitic growth in cultured cells, a biphasic morphology of electron dense and reticulated forms, size comparable to that of all known Chlamydiae, and rDNA sequence comparisons(32-36). The S. negevensis full length 16S and 23S rDNA sequences are each 80-87% identical to those of members of the Chlamydiaceae, whereas all members of the Chlamydiaceae have >90% identity with each other. Although the full genome sequence of S.negevensis has not been determined, partial DNA sequences of the RNase Pgene (rnpB) , mutS, and groEL have been determined from PCR amplified products using primers based on consensus sequences determined from several strains of chlamydia and other bacteria. Taxonomic relationships among chlamydiaceae species have been confirmed by analyses of GroEL (the 60-kDa heat shock protein), KDO transferase, the small cysteine-rich lipoprotein, the 60 kDa cysteine-rich protein, and the major outer membrane protein (MOMP)(21,22).

Antigens and membrane structure

The polypeptide pattern of simkania negevensis is significantly different from that of other members of the chlamydiales. Only a relatively small number of common epitopes (most likely heat shock proteins) are shown to be shared when antigen panels of S.negevensis, Waddleia chondrophila, Parachlamydia acanthamoebae (23,24,25,26,27), C.trachomatis and Chlamydophila (Chlamydia) pneumonia (28,29,30,31) are tested for cross reactivity by western blot using specific polyclonal antisera (36,37,38,39,40).

Immunoblot S.negevensis whole cell lysates and sarcosyl insoluble membrane preparations using hyperimmune murine sera showed the following predominant membrane fraction antigens: asset of three bands migrating at 37-42kDa , additional bands at 26,52,58, and 64 kDa , and a slower migrating band at >100 kDa. Monoclonal antibodies (MAbs) prepared against whole organisms or whole cell lysates reacted mainly with the 37-to 42 kDa set of bands, indicating that the complex was strongly immunogenic. Some of these MAbs were capable of neutralizing S.negevensis in vitro, but were unable to neutralize C.trachomatis under the same conditions.

When these MAbs raised against S.negevensis were tested by immunoblotting on C.trachomatis whole cell lysates or membrane preparations, they were able to recognize an epitope located on the OmpA protein. On the other hand ,Abs recognizing family-specific epitopes of chlamydial OmcB (OMP 2), OmpA(MOMP), and LPS did not bind S Negevensis antigens. The structure and the function of the three polypeptides belonging to the S negevensis outer membrane complex need to be further investigated. It may be that shared epitopes constitute part of a porin structure common to the Chlamydiaceae and S negevensis (40.41,42).

Growth life cycle

Simkania replicates in a biphasic life cycle similar to *Chlamydia*. Infection starts with an electron dense elementary body (0.2-0.3 m), which differentiates into the electron lucent replicative form called the reticulate body (0.3-0.7 m). The active replication cycle of Sn takes about 3–5 days and extensive long-term relationship with the host cell of about 10–15 days has been reported (Kahane et al, 2002). The strategy that this pathogen takes to keep its host alive for such a long period of time is unknown. Unlike other *Chlamydiales* (Kahane et al, 2002) observed that in Sn the replicative form may also be infective. This assumption was derived from the fact that they could observe similar kinetics of growth curve in less dense (RB) and more dense (EB) urografin fractions.

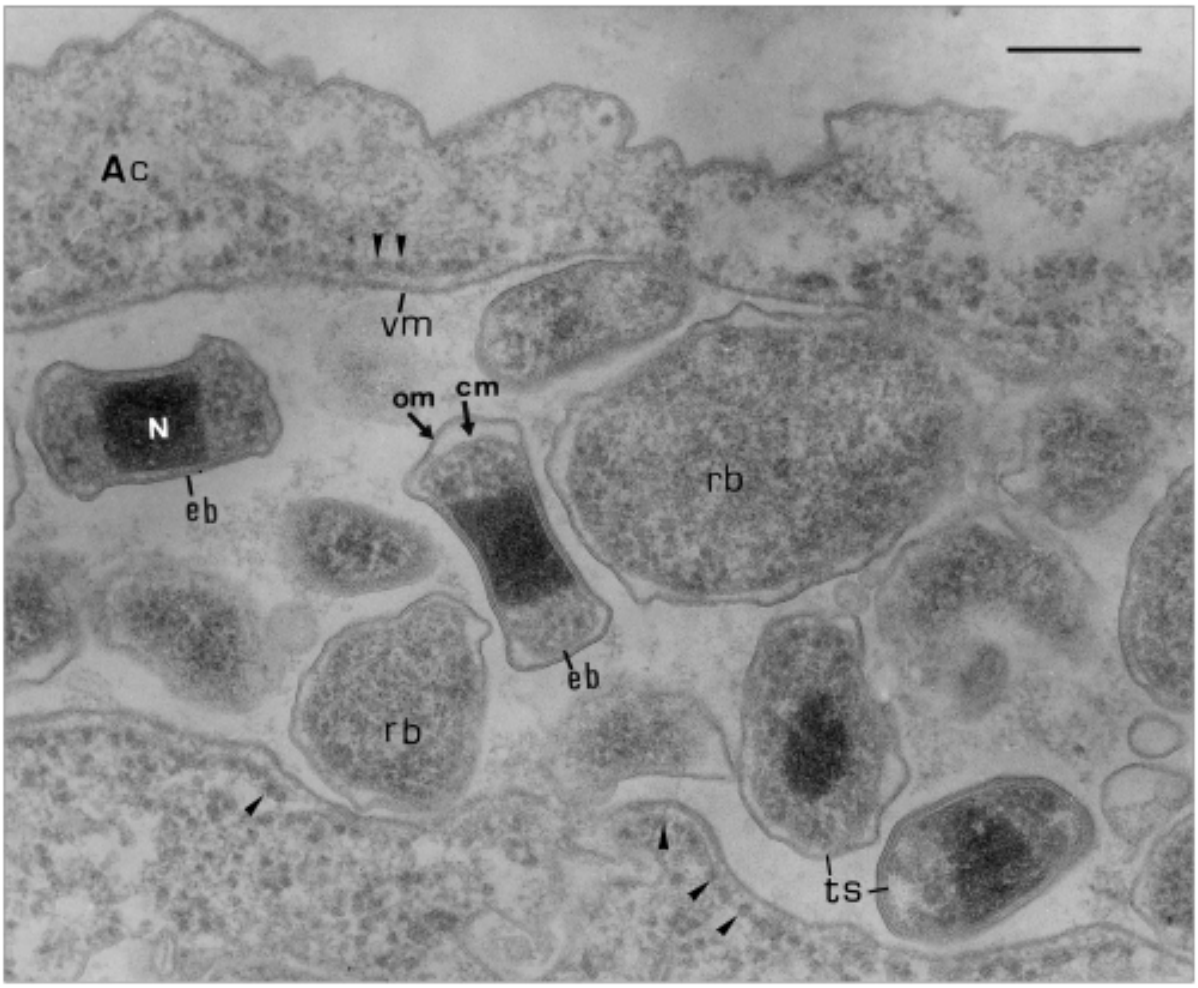
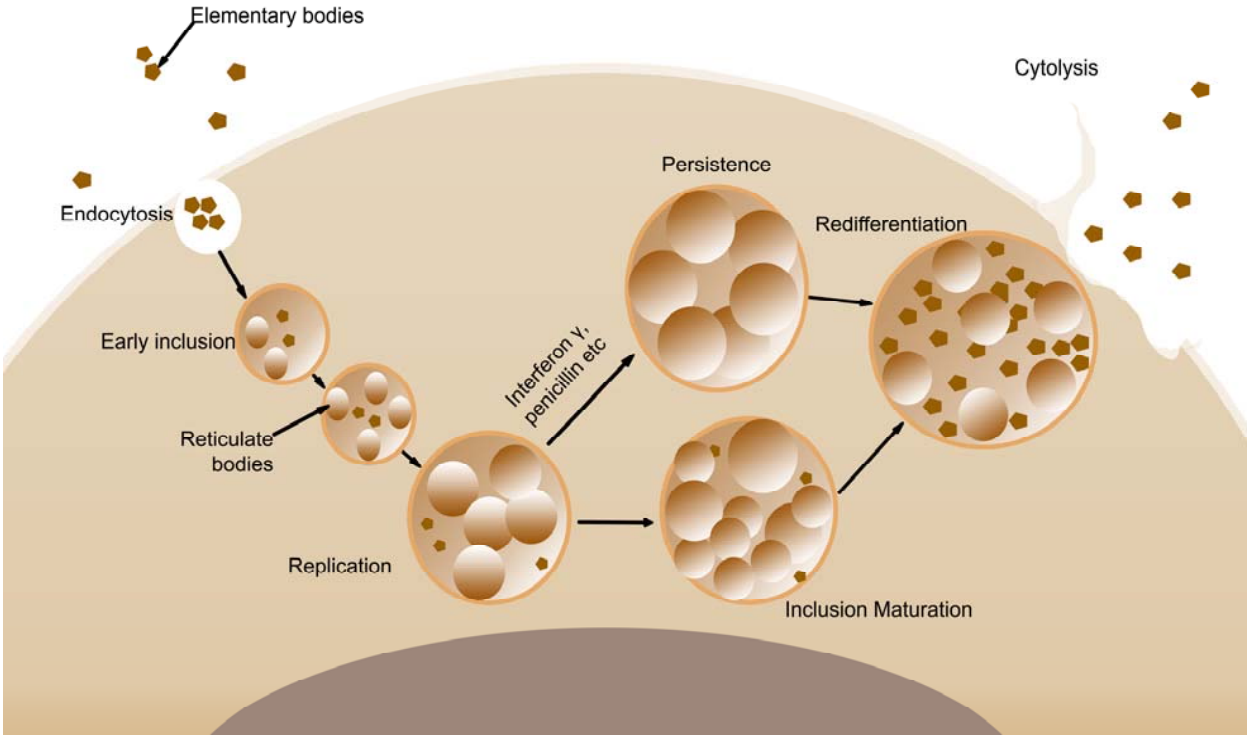


fig 1 **.** Detail of an *Acanthamoeba* trophozoite (Ac) harbouring different developmental stages of *Simkania* replicating within a membrane- bound vacuole (vm). The elementary bodies (eb) contain electron-dense nuclear material (N) and ribosomes. They are surrounded by the outer membrane (om) at some places separated from the inner cytoplasmic membrane (cm). They are considered as Gram-negative. The significantly larger reticulate stages (rb) have less dense fibrillar nuclear material and more ribosomes. They are also surrounded by an obviously more flexible trilaminar envelope. Transitory stages (ts) show intermediate traits between EBs and RBs. Remarkable are distinct ribosomes within the host's cytoplasm which appear arranged like a string of pearls beneath the vacuolar membrane (arrowheads). Scale bar 0.25 μm.

For decades research progress has been hampered to fully understand the genetics of *Chlamydiales* as they are obligate intracellular pathogens, there is no means to genetically manipulate them. Recently (Wang et al, 2011) had discovered that foreign DNA could be introduced into the chlamydial inclusion by using calcium ions. This will pave way for the chlamydial genetics and could lead to the development of chlamydial vaccines and therapeutic interventions.

Chlamydiales have a unique biphasic life cycle. Bedson and colleagues first observed these distinct particles when they examined psittacosis-infected tissue (Bedson et al 1932). Later the existence of these developmental forms confirmed under electron microscopic studies (Constable, 1959; Gaylord, 1954). The pathogen exists in two morphological forms namely elementary body (EB), which is infective, but metabolically inactive and reticulate body (RB), metabolically active and non- infective. The EB is 0.3 μm in diameter and RB around 1 μm. Infection is initiated by adherence of EB to the host cells through an unknown receptor that probably binds with heparan sulfate-like glycosaminoglycan present on the cell surface (Gutierrez- Martin et al, 1997; Kuo et al, 1973). Several other ligands for *Chlamydia* binding have also been proposed, including Hsp70 and Omp2 (Joseph & Bose, 1991).

Recent studies further revealed the crucial role of the cellular PDGF-receptor, the host kinase c-Abl, and the chlamydial protein Tarp (translocated actin recruiting phosphoprotein) in pathogen uptake (Clifton et al, 2004; Elwell et al, 2008). Together with other chlamydial effector proteins Tarp is translocated into the host cell via a type III secretion system (T3SS), a needle-like protein complex that spans the bacterial membranes (Subtle et al, 2000). Cytoskeleton rearrangements induce the uptake of the EB, which can also be seen in non-phagocytic cell (Jewett et al, 2008). After binding, the *Chlamydia* is internalized, enveloped within membrane-bound compartments that are subjected to bacteria induced modifications both in their luminal environment and in their membrane composition, and transported to a perinuclear location. These modified *Chlamydia*-containing membrane compartments are termed inclusions. The bacteria appear to survive within lysosomes (Eissenberg et al, 1983; Scidmore et al, 1996a; Scidmore et al, 1996b) whereas the the inclusion in host epithelial cells through their ability to inhibit fusion between the inclusions and fusion to sphingomyelinrich vesicles is enhanced (Hackstadt et al, 1995).



F*igure 2 - Infectious EB adhere to the receptors on the host cell. Upon internalization, the chlamydial inclusion is formed where EB differentiate into RB, which replicate by binary fission to produce several hundred particles that re-differentiate into infectious EB which leave the cell in order to start a new round of infection. Under certain conditions, such as sub-lethal doses of antibiotics like penicillin, IFN-gamma stimulation or co-infection with virus, aberrant bodies can be formed to persist inside the host cell. Depending on the species, the developmental cycle is completed after 48-72 h or 5-8 days in tissue culture cell lines.*

Within the inclusions, the EB differentiates into RB, which synthesize proteins and their own DNA and RNA then replicate by binary fission to form micro-colonies within the inclusion bodies. Differentiation of an EB into RB involves an increase in size, reduction of disulphide links in the outer membrane, unraveling of DNA in the condensed nucleoid and appearance of granular ribosomes (Wang et al, 1994). The initial signal for the differentiation of EB into RB is not known. By 18 hours postinfection (hpi), a proportion of the developed RB begins to transform back into EB

while the remainder continues to divide in the growing inclusion (Hackstadt et al, 1999) within 24 h large inclusions are formed. Between 24 and 72 hpi the RB redifferentiate into EB and are released into the extracellular space by rupturing the cells to start a new round of infection (Moulder, 1991) . As the RB is not infectious, re-differentiation into EB is an essential step in the chlamydial developmental cycle for the establishment of an acute infection. The engagement of the chlamydial type III secretion system in RB to EB re-differentiation was recently discussed by (Peters et al, 2007). The environmental signal that induces the reorganization from RB to EB is undefined. In the presence of Interferon , virus infection or antibiotics like penicillin the chlamydial particles gets stuck reversibly in the persistence stage where only aberrant bodies are observed. Persistence is the viable but non-cultivable growth stage of *Chlamydia* where it maintains long term relation with the host cells (Hogan et al, 2004). Depending on the species and the amount of bacteria that entered the cell, the developmental cycle of *C. trachomatis, C. pneumonia* and *Simkania negevensis* in cultured cell lines is completed after 48 h, 72 h and 5 days, respectively.

Antibiotics and cytokine sensitivities

Although in its sensitivity to macrolides and tetracyclines S negevensis is similar to the chlamydia, unlike these cousins, it is totally resistant to penicillin, and indeed, is routinely grown in the presence of penicillin. While penicillin and other beta lactams are not the drugs of choice for treatment of chlamydial infections, in vitro the organisms are sensitive to the presence of these drugs, and their development is inhibited at the reticulate body stage in the presence of beta lactams. S negevensis was found to be resistant to bacitracin, but sensitive to D-cycloserine; both these drugs are inhibitors of peptidoglycan synthesis. Hybridization experiments using cloned C trachoma tis penicillin binding protein DNA as probes indicated the presence of homologous DNA in S negevensis. Whether S negevensis “penicillin binding proteins “ are not transcribed or have no penicillin binding sites still remains to be investigated. preliminary experiments indicate that azithromycin, minocycline and erythromycin are the most effective inhibitory and bactericidal drugs in cycloheximide treated Vero cells.

S.negevensis is resistant to high concentrations of interferon gamma when grown in HEp-2 cells and inhibited by concentrations of tumor necrosis factor (TNF alpha)(43,44,45).

Seroprevalence

The seroprevalence of S. negevensis in the population shows significant variations in different parts of the world, where it is seen as a high percentage of 55-80% in Israel, but also a low percentage as 4% in Japan. These differences may be partly due to the sensitivity and specificity of the approaches used serological and the cut-off for positivity established (46). The involvement of S. negevensis in respiratory tract infections has been studied in different cohorts of patients in Europe, the Middle East and America using serological or molecular diagnostic methods. Some of these studies report an association between S. negevensis and community-acquired pneumonia (CAP), exacerbation of chronic obstructive pulmonary disease and (COPD) and bronchiolitis in adults and children .

The role of this bacterium as the causative agent of CAP is, however, difficult to assess and, for this reason, further work is needed to clarify the epidemiological and serological importance of this organism at the clinical level.

The incidence of S. negevensis could be higher than that observed in some populations or ethnic groups, in the presence of concentrations of the microorganism undetectable to the diagnostic methods used. It was documented a high seroprevalence of S. negevensis, in studies in the Middle East (the Bedouins) and in northern Canada (in Inuit).

A study carried out in Brooklyn (NY), in contrast, did not show any association of S. negevensis with respiratory problems, despite the high prevalence (23.5%) of antibodies against S. negevensis in adults and children of this population, assuming that these bacteria like Chlamydia are simple colonizers (diners). Therefore, a thorough assessment of the pathogenic role of S. negevensis is necessary (47).

It was also studied the possible involvement of S. negevensis other breathing disorders, such as chronic cough or asthma, but the data in this regard are still doubts .

In one study, S. negevensis was sought through the molecular method of PCR in samples of bronchoalveolar lavage of patients undergoing lung transplantation, showing surprisingly high prevalence of this microorganism of 97.5% (compared to 14% in patients highlighted undergoing transplantation of other solid organs) with a possible association with acute rejection. In fact, 59%

of patients positive in PCR, for S. negevensis, in comparison to 30% of patients with rejection but results PCR negative for S. negevensis (48).

A further study highlights the fact that hospitalized patients exposed to aerosol particles, such as mechanical ventilation, could thus be colonized or infected by this intracellular bacterium, whose pathogenic role in this environment was investigated at the time, very little .

They are very interesting studies performed in vitro to understand the pathogenesis of infections with S. negevensis: in these studies was demonstrated the ability of the microorganism infecting human macrophages and induce an inflammatory response (48).

Infections on human

Despite advances in antibiotic therapy, pneumonia remains one of the leading infectious causes of death in developed countries and a major cause of morbidity, especially in the elderly population and among patients with chronic underlying diseases (Mandell et al., 2007). Although a broad spectrum of microbial pathogens have been recognized as causal agents of respiratory tract infections (RTIs), the offending microorganism remains unknown in about half of the cases of community-acquired pneumonia (CAP) (Bochud et al., 2001; Echols et al., 2008), and three quarters of the cases of nosocomial pneumonia (Costa et al., 2001).

Microorganisms causing pneumonia may be acquired from respiratory droplets through human-to-human con- tact or from aerosolized particles from an animal or environmental reservoir. The epidemiology of RTI thus strongly depends on the interactions between humans and their ecosystem and evolves according to environmental changes due to human activities, climatic or ecological perturbations. Pandemics of influenza or the emergence of new respiratory diseases such as severe acute respiratory syndrome are dramatic illustrations of these phenomena.

Free-living amoebae live in water, soil and at the water–air interface. As they generally use bacteria as their main nutritional source, they are especially present in large quantities in sediments and biofilms (Rodriguez-Zaragoza, 1994). Thus, humans have been increasingly exposed to amoebae and to their related bacterial pathogens with the progressive development of various modern man-made water systems such as water-treatment plants, cooling towers, air conditioners, humidifiers, spas and swimming pools. Apart from L. pneumophila, which has mainly been recognized due to the dramatic importance of the Philadelphia outbreak, many other bacteria that resist the phagocytic

amoebae may also use these protists as widespread reservoirs and may have acquired virulence traits promoting their resistance to macrophages. Interestingly, some of these amoebae-resisting bacteria have also been discovered during outbreaks of RTI (Herwaldt et al., 1984; Birtles et al., 1997). Indeed, given their intracellular lifestyle, they either do not grow or only poorly grow in conventional axenic media. It is important that the microbiology community be well aware of these new emerging human agents of pneumonia and develop new diagnostic tools for their identification. Amoebal coculture and amoebal enrichment coupled with detection of potential intra-amoebal bacteria have been demonstrated to be largely successful in identifying a large biodiversity of new pathogens from patients (Corsaro et al., 2009).

Simkania negevensis may easily grow within the Acanthamoeba amoeba and may also use free-living amoebae as a widespread environmental reservoir. Simkania negevensis was first described in 1993 as a cell culture contaminant of unknown origin exhibiting a typical two-stage developmental cycle, with infectious elementary bodies and replicative reticulate bodies, but differing significantly from Chlamydiaceae (Kahane et al., 1993). Its seroprevalence in the population displays important variations around the world, having been reported to be as high as 55–80% in Israel and only 4% in Japan. These differences may be partially due to the respective sensitivity and specificity of the serological approaches used and to the cut-off defining positivity. The involvement of S. negevensis in RTIs has been investigated in several large cohorts of patients in Europe, the Middle East and America using serological or molecular diagnostic methods (Nascimento- Carvalho et al., 2009). Some of these analyses reported an association with CAP, exacerbations of chronic obstructive pulmonary diseases or bronchiolitis in adults and children. The importance of this bacterium as a causal agent of CAP is, however, difficult to evaluate and seems relatively marginal (o 2% of all aetiologies) in view of these results. Its incidence may be higher in some populations or ethnic groups where a high seroprevalence of S. negevensis has been documented, for example in the Middle East (Bedouins) or Northern Canada (Inuits) (Greenberg et al., 2003), whereas it remains to be determined in many other parts of the world. A study carried out in Brooklyn (NY) deserves mention as it did not identify any association with respiratory diseases despite a high prevalence (23.5%) of antibody titers against S. negevensis among adults and children in this population, suggesting that these Chlamydia-related bacteria are simple colonizers. Thus, thorough evaluation of the pathogenic role of S. negevensis is warranted, as most studies did

not include a control group, and among the few studies with a control group, most failed to demonstrate a significant correlation with lower RTIs.

The implication of S. negevensis in other respiratory diseases, such as chronic cough or asthma, has also been investigated and could not be demonstrated conclusively (Korppi et al., 2006). In one study, S. negevensis was detected by a PCR method in bronchoalveolar lavage samples of lung transplant recipients with a surprisingly high prevalence (97.5%, when compared with only 14.1% in other solid-organ transplant recipients; P o 0.0001) (Husain et al., 2007). Many of these patients did not have documented pneumonia and the pathogenic role of S. negevensis in this context thus remains unclear. The authors of this study postulated a possible role in acute graft rejection, although the analysis was underpowered to reach statistical significance. Like other Chlamydia-related organ- isms, S. negevensis may infect free-living amoebae such as Acanthamoeba which are widespread in water, including hospital water supplies (La Scola et al., 2002). Hospitalized patients exposed to aerosolized particles, such as those undergoing mechanical ventilation, may thus be colonized or infected by these intracellular bacteria, whose pathogenic role in this setting has been poorly investigated. However, it is worth noting that in vitro studies of the pathogenesis of S. negevensis have demonstrated its ability to infect human macrophages and to induce a host cell inflammatory response, supporting its potential ability to cause human infections and the need for further clinical investigations.

table 1_ Simkania negevensis as causal agents of pneumonia: review of literature.

Population (country) and number of patients	Disease	Diagnostic method	Positive results	*P* value (if controls)	Recent infection* and no alternative pathogen	References
Adults (Israel) 308	CAP	Serology (IgG, IgA)	112 (37%)	No control	4 (1.3%)	Lieberman *et al*. (1997)
Infants (Israel) 239	Bronchiolitis	Culture and/or PCR (NP swabs)	60 (25%)	$P < 0.001$	38 (16%)	Kahane *et al*. (1998)
Adults (Israel) 190	COPD exacerbation	Serology (IgG, IgA)	120 (63%)	Not significant	1 (0.5%)	Lieberman *et al*. (2002)
Infants (Canada) 22	Bronchiolitis	PCR (NP swabs)	14 (64%)	No control	2 (9%)	Greenberg *et al*. (2003)
Adults/children (USA) 188	Bronchiolitis, Pneumonia	Serology (IgG) ($n = 69$)	14 (18%)	Not significant	NA	Kumar *et al*. (2005)
	Asthma	PCR (NPl swabs) ($n = 169$)	29 (17%)	Not significant	NA	
Adults (UK) 29	RTI	Serology (IgG)	18 (62%)	Not significant	NA	Friedman *et al*. (2006)
		Serology (IgA)	5 (17%)	$P = 0.004$	NA	
Children (UK) 222	Bronchiolitis	Culture and/or PCR (NP swabs)	111 (50%)	No control	NA	Friedman *et al*. (2006)
Children (Italy) 101	CAP	Serology (IgM, IgG)	20–30%	No control	2 (2%)	Fasoli *et al*. (2008)
Children (Finland) 174	CAP	Serology (IgM)	18 (10%)	No control	6 (3.4%)	Heiskanen-Kosma *et al*. (2008)
Children (Brazil) 184	CAP	Serology (IgM, IgG)	3 (1.6%)	No control	1 (0.5%)	Nascimento-Carvalho *et al*. (2009)

*Recent infection was defined as: serological evidence for recent infection (positive IgM or significant increase between initial and convalescent IgG titres) or positive PCR result during the course of infection.
COPD, chronic obstructive pulmonary disease; NA, data not available.

Pathogenic potential of emerging chlamydiae

Free-living amoebae such as Acanthamoeba and Hartmannella play a key role as reservoirs and/or vectors for a variety of amoeba-resisting microorganisms (49), including the chlamydiae. Acanthamoeba is a suitable host for most Parachlamydiaceae (50,51) and S. negevensis (52,53), whereas Hartmannella, Balamuthia, Dictyostelium, and Naegleria are also able to sustain infection by some Parachlamydiaceae (54,55), S. negevensis (56), and Waddlia chondrophila (55). Various interactions between chlamydiae and amoebae may take place, depending on the chlamydial and the amoebal species and strain and on environmental conditions. Thus, P. acanthamoebae is endosymbiotic at 25 to 30°C and lytic at 32 to 37°C for Acanthamoeba spp.. Since the temperature of the human nasal mucosa is generally lower (about 30°C) than that of the lower respiratory tract (generally between 35 and 37°C), P. acanthamoebae could reside symbiotically within amoebae present on the nasal mucosa and induce the lysis of the amoebae that reach the lower respiratory

tract, inducing their own release and possibly starting an infectious process. Conversely, the virulence of the amoebae could be increased when harboring chlamydiae.

Free-living amoebae, mainly Acanthamoeba spp., have been shown to be natural hosts for several amoeba-resisting bacteria, including Parachlamydiaceae. These unicellular eukaryotes have also been successfully used in a cell culture system to isolate a variety of amoeba-resisting bacteria from clinical samples .

The fact that C. pneumoniae and S. negevensis are able to infect Acanthamoeba in vitro and the fact that free-living amoebae likely play a role as an environmental host of Simkania support the use of Acanthamoeba to isolate novel chlamydiae from clinical samples. Of note, Neochlamydia hartmannellae (but not other Neochlamydia spp. , has been successfully grown within Hartmannella vermiformis but not within Acanthamoeba. Moreover, Waddlia chondrophila may grow in Acanthamoeba and other free-living amoebae, but only after subcultivation in H. vermiformis. Efforts to cultivate Fritschea in Acanthamoeba have failed. In addition, experimental studies have shown that a wide diversity of free-living amoebae are able to sustain the growth of the new chlamydiae and not all strains of a free-living amoeba species are susceptible to infection by these agents. This should be taken into account when interpreting negative coculture results. Thus, the use of more than one strain of Acanthamoeba and/or the use of several amoeba species (e.g., Acanthamoeba and Hartmannella) is recommended to increase the rate of isolation of chlamydiae, at least from clinical samples that give a positive PCR result. In addition to the possible problem of a persistent viable but noncultivable stage, false-negative culture may also occur due to the use of a cell that is not permissive to a given species (i.e., Neochlamydia and Acanthamoeba) or due to the presence of antibiotics in the amoebal coculture to which the bacterium is susceptible. False-positive results might occur if the cell line is contaminated with an endosymbiotic chlamydia (which will not be easily detected) or if the broth is not adequately filtered and/or heat sterilized.

Since amoebae may harbor Parachlamydiaceae, S. negevensis, and Waddlia chondrophila , it may be useful to first isolate amoebae from clinical samples and then look within these amoebae for whether a chlamydia is present. Screening of environmental samples for the presence of amoebae may also contribute to the identification of a possible source of infection (e.g., humidifiers or air conditioning systems). Amoebal enrichment may be performed using established protocols

Specimens are generally seeded on nonnutrient agar preinoculated with heat-inactivated Escherichia coli.

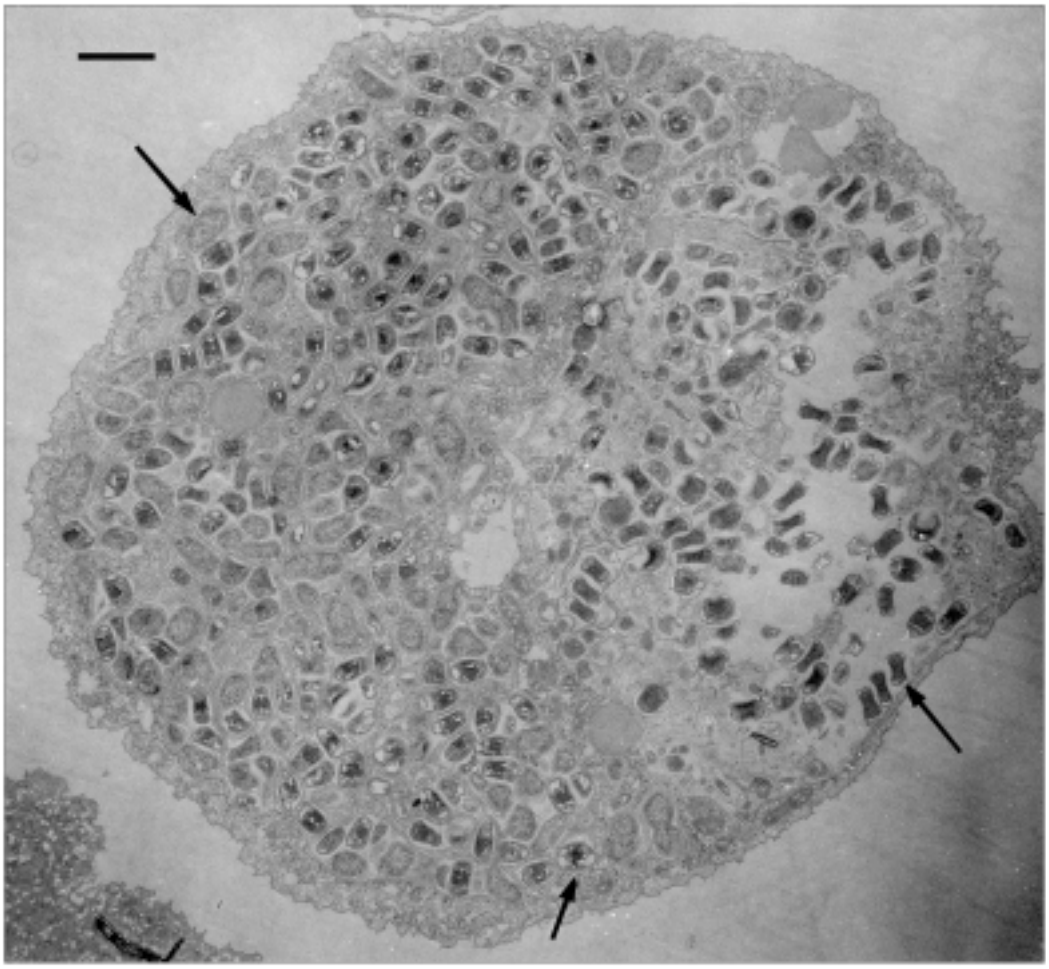


fig 3 overview of an infected Acanthamoeba, strain HLA, jammed with numerous organisms of simkania negevensis (arrows) as a result of multiplication of the endoparasite in a period of six days. Scale bar 1.0 microm.

Aim of the project

Intracellular bacterial pathogens may have a variety of relationships with their hosts, leading either to acute or to chronic infections, and resulting in short term or long term pathologies. A novel approach to learning more about the potential pathogenesis of S.negevensis is to examine in vitro which types of epithelial cells it is able to infect, and which of these it may activate to inflammatory citokine secretion(56).

Because of its ability to assume persistent forms of infection, which may lead to a prolonged inflammatory response, Simkania, may be involved in pathologic complications.

Free-living amoebae such as *Acanthamoeba* and *Hartmannella* play a key role as reservoirs and/or vectors for a variety of amoeba-resisting microorganisms, including the chlamydiae.

Since amoebae may harbor *Parachlamydiaceae*, *S. negevensis* , and *Waddlia chondrophila*, it may be useful to first isolate amoebae from clinical samples and then look within these amoebae for whether a chlamydia is present. Screening of environmental samples for the presence of amoebae may also contribute to the identification of a possible source of infection (e.g., humidifiers or air conditioning systems).

Transmission of SN is probably by droplet or close contact, but there is evidence that water supplies are also implicated, and this was subject of this study (currently under way). According to a recent study (57) was showed a seroprevalence of IgG and IgA anti S. negevensis significantly higher , not only in patients with respiratory diseases , but also in patients with gastrointestinal disorders compared to the healthy population. Since S. negevensis survives and multiplies within amoebae and has been highlighted in the sources and collected water , has been suggested for patients we studied a way of oral transmission of the infection, in addition to that already known via aerosol or closely.

The main purpose of this study was to evaluate the impact of S. negevensis population of hemodialysis patients and carriers of kidney transplant, to study correlations between clinical events (gastrointestinal and respiratory symptoms) and positive test for the infection, search correlation between infection and laboratory indices (indices of nonspecific inflammatory and non-differential, transaminases).

On the subject of transplantation is also important to open a debate on a worldwide basis over the fact that the infection can be S.negevensis, as other pathogens, closely related to the acute rejection of transplanted lungs.

Samples and methods

We studied 111 patients with kidney transplants, and 112 patients undergoing hemodialysis. Were collected the personal data, age, sex, height, weight, the underlying disease for which there has been the chronic renal failure.

From the laboratory point of view were collected such data: the values of creatinine, the presence or absence of proteinuria, hemoglobin values, transaminases, complete blood count with differential, the non-specific inflammatory markers (ESR, CRP). It was collected complete clinical history .

Were investigated any respiratory and gastrointestinal symptoms at the time of withdrawal.

Have been investigated IgG and IgA anti Simkania negevensis in serum samples of patients on dialysis and patients undergoing kidney transplantation.

Were analyzed waters network used for hemodialysis before and after the treatments disinfectants: sampled and tested for the presence of S.negevensis .

Cultivation of C. pneumoniae and S. negevensis

The reference strain of S. negevensis ZT (ATCC VR- 1471) and the reference strain IOL- 207 of C. pneumoniae were grown in LLC - MK2 cells (continuous line coming from monkey kidney) , cultured in sterile flasks in the presence of soil , Eagle MEM (Minimal Essential Medium) .

For the culture of cells were added to a series of substances to the soil , such as fetal bovine serum (SBF) , glucose, L - glutamine and also antibiotics and antifungal agents (to prevent the growth of other bacteria and / or fungi) as vancomycin , gentamicin and fungizone(inactive against the two microorganisms) .

To encourage the growth of S. negevensis and C. pneumoniae to the growth medium , containing higher concentrations of drugs , glucose and SBF respect to the ground for cell culture , cycloheximide has been added that , by slowing down the cellular metabolism , increases the infectivity elementary bodies (33) . The concentrations of the various substances added to soil is shown in Table .

	Terreno Eagle MEM per cellule (ogni 500 ml)	Terreno Eagle MEM per *C. pneumonia e* (500 ml)	Terreno RPMI per *S. negevensis* (500ml)
SBF (inattivato a 56°C per 30 minuti)	50 ml	100 ml	75 ml
L-glutammina (200mM)	5 ml	5 ml	5ml
Glucosio	6 ml (5,94 gr/100 ml H_2O)	2 gr	4 g
Fungizone	0,15 (µg/ml)	1 ml (1 µg/ml)	1 ml (2 µg/ml)
Vancomicina	0,5 ml (10µg/ml)	5 ml (10µg/ml)	8 ml (160 µg/ml)
Gentamicina	0,125 ml (40µg/ml)	0,6 ml (40µg/ml)	1,2 ml (100 µg/ml)
Cicloeximide (1*µ*g/ml)		0,7 ml	0,7

For S. negevensis was used RPMI (Roswell Park Memorial Institute) and C. pneumoniae soil Eagle MEM (Minimal Essential Medium) .

The flasks containing the cell suspensions were incubated for 48-72 hours in a thermostat at 37 ° C in the presence of 5 % CO2. After the formation of the monolayer , the cells were detached from the flask with trypsin - EDTA and resuspended in growth medium and again , so as to obtain a concentration of 100.000 cells / ml . The cell suspension was distributed in plastic tubes with screw cap , containing a glass slide on the bottom of 1 cm in diameter , in the amount of 1 ml of cell suspension / tube. The test pieces were put back into the incubator so as to obtain 100,000 cells / slide after about 24 hours of incubation . The next day , the tubes were infected with C. pneumoniae and S. negevensis . Before infecting the cell monolayer was removed the growth medium of the cells and replaced with 1 ml of suspension of S. negevensis or C. pneumoniae . To facilitate the infection , and the entrance of micro-organisms within the cells , the tubes were centrifuged at 3000 rpm for 3 hours , at a temperature of 33 ° C and then placed to incubate in a thermostat at 37 ° C in the presence of 5 % CO2 . Given the different duration of the development cycle of C. pneumoniae and S. negevensis , the monolayers infected with C. pneumoniae have been made incubate for 3

days while those infected with S. negevensis for 5 days. To check the degree of infectivity was performed by direct immunofluorescence staining in the case of C. pneumoniae and indirect for S. negevensis .

In this regard , the soil was removed accretion inside of the tubes and replaced by methanol , which was left on for 5 minutes to allow the attachment of the cells .

For direct staining of C. pneumoniae , the monolayer was put into contact with monoclonal antibodies directed against the LPS of chlamydia , conjugated to fluorescein , and left to incubate for 30 minutes in pan moist at 37 ° C . After incubation has been carried out a quick wash in distilled H2O and the slides were mounted with glycerin. For staining of S. negevensis , instead , the monolayer , always after fixing with methanol , was put in contact with the monoclonal antibody D5-14 produced in our laboratory and reactive against a surface protein of 64-66 kDa S. negevensis . After an incubation for 40 minutes in pan moist at 37 ° C , were carried out washing with H2O and PBS , and then was added the secondary antibody anti - mouse immunoglobulins conjugated with fluorescein diluted 1/30 . Incubation for 30 minutes in pan moist at 37 ° C , followed by additional washes and finally to the monolayer was added Evans blue for a minute so as to dampen the background fluorescence and the slides were mounted with glycerin. At this point it was possible to monitor fluorescence microscopy infectivity in cell monolayers , S. and C. pneumoniae negevensis , noting the number of green fluorescent cytoplasmic inclusions present compared to the cell nuclei and uninfected cells stained red. The evaluation of the degree of infectivity (number of inclusions present) allows to calculate the appropriate dilution of S. negevensis or C. pneumoniae in such a way as to obtain , in the next step into new cells , the 100 % of infectivity.

At this point , to obtain large quantities of cells infected with C. pneumoniae or S. negevensis , the tubes with the infected monolayers were stirred for at least 1 minute vortexing (in order to detach the monolayer from the slide) and the suspension with infected cells , appropriately diluted with the suitable medium according to the microorganism , was inoculated in 30 -well plates at 6 to the bottom plane containing cell monolayers , in the amount of 4 ml / well . The plates were then placed in centrifuge at 2000 rpm for 2 hours at 33 ° C and subsequently left in an incubator for 3 or 5 days to allow growth of C. pneumoniae or S. negevensis respectively (58,59,60).

Purification of the elementary bodies of C. pneumoniae and S. negevensis

The elementary bodies (EC) of S. pneumoniae and C. negevensis were purified sucrose gradient following the procedure Fukushi and Hirai , with appropriate modifications. After 3 or 5 days post infection , the infected cell monolayer was detached from the plates using sterile rubber and collected in 50 ml tubes , and kept in ice. The suspension was sonicated 3 times at low intensity for 15 seconds , so as to break the cell membranes and release the micro-organisms and then centrifuged at 3000 rpm for 10 minutes at 4 ° C . The supernatant recovered , this has been allocated to ultracentrifuge tubes and centrifuged at 18,000 rpm for 35 min at 4 ° C , with maximum acceleration and deceleration . Once the pellet , removing the supernatant , it was resuspended in 40 ml total Tris buffer (0.01 M , pH 7.4) , sonicated two times for 15 seconds and layered over a cushion of 8 ml of sucrose to 30 % in Tris Buffer , and then ultracentrifuged at 18000 rpm for 40 minutes at 4 ° C , with minimum acceleration and deceleration . After centrifugation the pellet was resuspended in 2 ml of Tris buffer , sonicated 1 time for 10 seconds and placed gently on 30-60 % sucrose gradient in Tris . The gradient was previously obtained by slowly passing , with a peristaltic pump , 14 ml of 60 % sucrose solution in TRIS and 14 ml of the 30% in a test tube to ultracentrifuge . The subsequent centrifugation was carried out at 18000 rpm for 50 minutes at 4 ° C , with minimum acceleration and deceleration . The gang of EC was then collected with a syringe , and resuspended in about 30 ml of Tris then centrifuged at 18000 rpm for 35 minutes at 4 ° C , with maximum acceleration and deceleration . The EC present in the pellet were resuspended in 250-300 L of Tris buffer , sonicated 1 time for 10 seconds , divided into small aliquots of 10 l each and stored at -70 ° C until use .

Enzyme immunoassay ' home -made '

The enzyme immunoassay we used was that developed previously for the detection of antibodies against C. psittaci (35). The protein concentration of the EB was determined using the Bio-Rad protein assay kit (Bio-Rad , Richmond, CA) with bovine serum albumin as a standard. The EC of elementary S. negevensis or C. pneumoniae , previously purified , were diluted , in order to obtain a final concentration of 1 mg / ml in Coating Buffer PBS with 0.03M 0.068M Sodium Carbonate and Sodium Bicarbonate , pH 9.4-9.8) and dispensed in amounts of 100 µl / well in a 96 well microplate.

The plate was kept overnight in pan moist at 4 ° C . The next day , the plate was turned upside down to empty the wells and were added to each well 200 L of blocking solution of 5% milk in PBS -Tween - EDTA . After incubation for 1 hour at room temperature , 5 washes were carried out over a period of 10 min with 200 L of PBS-Tween - EDTA / well . Subsequently , were dispensed 200 L of 1% milk in PBS -Tween - EDTA to which were added 2 L of serum of patients to be examined so as to obtain a dilution of each serum of 1/100 for both research IgG that IgA anti - S . negevensis or C. pneumoniae .

The plate was then left in incubation for 1 hour at 37 ° C , and then washed again as described above . At this point , were added 100 L of a polyclonal serum anti - human IgG or IgA (produced in rabbits) conjugated with peroxidase and diluted 1/500 in PBS -Tween - EDTA in each well of the plate . After incubation for 1 hour at 37 ° C , were carried further washing and finally added to the wells 100 L of tetramethylbenzidine (substrate specific for the enzyme) . The reaction was finally stopped after about 10 minutes, adding to the wells 100 L of stop solution. The absorbance of the samples examined was read in a spectrophotometer at a wavelength (λ) of 450 nm against 620 nm reference .

A serum sample was defined as positive when presented a value in optical density of at least two standard deviations above the average of 20 samples negative .

In addition, two control sera testing positive MIF for S. negevensis and two for C. pneumoniae were included in each experimental session ELISA. Each assay was performed in duplicate (61).

Results

Table 1 shows the comparisons between patients positive and negative results with the test for simkania and laboratory data .

Analyzing the test for simkania (positive and negative) was observed statistical significance of positive than negative towards certain laboratory parameters : ESR , white blood cells and specifically the neutrophils (VES $p < 0.037$; GB $p < 0.038$; neutrophils $p < 0.018$) . Non-parametric test was performed according to the method of Kruskal-Wallis .

Totale soggetti	Simkania								Kruskal-Wallis, p-value
	Negativo				Positivo				
	Mean	Median	Minimum	Maximum	Mean	Median	Minimum	Maximum	
Kt/v	63.80	66.05	23	80	66.83	66.15	59	74	0.448
età	58.39	58.50	19	90					\
anni trapianto	6.67	4.00	1	30	10.56	12.00	1	22	0.118
Creatiniemia	3.96	1.89	1	15	5.23	4.70	1	11	0.135
Proteinuria	37.59	0.00	0	412	48.00	0.00	0	300	0.885
Hb	317.21	11.80	8	36.060	11.15	11.15	8	15	0.222
AST	17.04	15.00	4	140	13.53	13.00	6	29	0.066
ALT	14.61	12.00	3	64	11.95	10.00	2	33	0.055
VES	29.84	25.00	2	84	42.73	39.00	8	101	0.037
PCR	59.65	.30		3.660	1.05	.33		6	0.573
GB	6.39	6.09	2	14	7.98	7.29	3	23	0.038
N	3.71	3.42	1	9	5.05	4.50	2	15	0.018
L	1.72	1.47		4	1.66	1.47		4	0.804
M	.47	.45		1	.45	.45		1	0.713
E	98.51	.14		9.504	.23	.18		1	0.089

Table 1. Comparisons between subjects positive and negative subjects to Simkania

Nonparametric test because the distributions are not normal .

ESR : the medians differ statistically significantly (25.00 vs. 39.00)

GB : medians differ statistically significantly (6:09 vs. 7:29)

N : the medians differ statistically significantly (3:42 vs. 4.5)

Tab.2 Comparisons Simkania positive / negative

		Simkania		Chi_square, p-value
		Negativo	Positivo	
Tx_dialisi	Trapianto	100	10	0.007
		53.2%	28.6%	
	Dialisi	88	25	
		46.8%	71.4%	
SESSO	F	59	20	0.005
		32.2%	57.1%	
	M	124	15	
		67.8%	42.9%	
Sintomi	Nessun sintomo	140	25	0.566
		81.4%	73.5%	
	Sintomi respiratori	24	7	
		14.0%	20.6%	
	Gastro intestinali	8	2	
		4.7%	5.9%	

According to Table 2 comparing the two populations , transplanted and dialysis , in relation to the positive test laboratory for finding Simkania showed that the largest number of patients testing positive were hemodialysis (the figure was considered statistically significant $p < 0.007$) . Also checking this figure in relation to sex showed that test positive predominantly women (even this figure was considered statistically significant $p < 0.005$) . To conclude evaluating tests positive for simkania and comparing it to the presence or absence of symptoms at the time of collection was observed a greater number of patients to symptomatic (this figure , however, has not considered statistically significant p 0.566) .

Totale soggetti	Tx_dialisi								Kruskal-Wallis, p-value
	Trapianto				Dialisi				
	Mean	Median	Minimum	Maximum	Mean	Median	Minimum	Maximum	
Kt/v					64.43	66.05	23	80	\
età	52.89	53.00	28	77	68.48	71.50	19	90	<0.001
anni trapianto	7.03	5.00	1	30					\
Creatiniemia	1.75	1.50	1	9	8.29	8.10	3	15	<0.001
Proteinuria	38.47	0.00	0	412					\
Hb	12.50	12.40	8	16	677.85	10.10	8	36.060	<0.001
AST	16.62	16.00	6	53	16.44	12.00	4	140	<0.001
ALT	14.56	12.00	4	53	13.71	11.00	2	64	0.222
VES	21.16	17.00	2	63	42.15	41.50	2	101	<0.001
PCR	14.17	.16		960	108.21	.68		3.660	<0.001
GB	6.54	6.25	2	13	6.75	6.23	2	23	0.944
N	3.87	3.69	1	9	3.90	3.36	1	15	0.455
L	1.81	1.67		4	1.55	1.21		4	0.016
M	.48	.47		1	.44	.45		1	0.204
E	139.94	.11		9.504	.26	.17		1	<0.001

Tab.3 *Comparisons between transplant recipients and subjects on dialysis*

Nonparametric test because the distributions are not normal .

Age : medians differ statistically significantly (53.00 vs. 71.50)

Creatinine : medians differ statistically significantly (1.50 vs. 8:10)

Hb : medians differ statistically significantly (12:40 vs. 10:10)

AST : medians differ statistically significantly (16:00 vs. 12:00)

ESR : the medians differ statistically significantly (17.00 vs. 41.50)

PCR : the medians differ statistically significantly (0:16 vs. 0.68)

L : the medians differ statistically significantly (1.67 vs. 1:21)

E: the medians differ statistically significantly (0:11 vs. 0:17)

Comparing the total sample used for the study , patients on dialysis and transplants and analyzing certain parameters both laboratory data and medical history has shown that the average age was higher among patients on dialysis (this figure reached statistical significance $p < 0.001$) , the value of creatinine was higher among hemodialysis (in relation to the difference in expected condition) , the hemoglobin was higher among transplant patients (statistically significant $p < 0.001$) ; transaminases (GOT) higher in transplanted (statistical significance $p < 0.001$) ; both the ESR that PCR were higher in the patient on hemodialysis ($p < 0.001$) ; the proportion of lymphocytes and

eosinophils higher in transplanted ($p < 0.016$ and $p < 0.001$) . The non-parametric test was conducted according to the method of Kruskal-Wallis .

		Tx_dialisi		Chi_square, p-value
		Trapianto	Dialisi	
SESSO	F	29	50	0.011
		27.6%	44.2%	
	M	76	63	
		72.4%	55.8%	
Sintomi	Nessun sintomo	82	83	0.028
		88.2%	73.5%	
	Sintomi respiratori	9	22	
		9.7%	19.5%	
	Gastro intestinali	2	8	
		2.2%	7.1%	

Table 4 Comparisons Transplant / Dialysis

Sex: the percentages of males differ statistically significantly between transplant and dialysis . Males tend to be more rather than transplanted dialysis .

From data collected the sample showed a greater number of patients transplanted male ($p < 0.001$), and most of the patient did not show any signs of disease especially kidney transplants ($p < 0.011$) and in the remaining part of the cases respiratory symptoms (prevailing in the dialysate 19.5 % vs 9.7 %) , and gastrointestinal (7.1 % vs 2.2 %) . This study was performed according to the method Chi square .

SOLO TRAPIANTATI	Simkania								Kruskal-Wallis, p-value
	Negativo				Positivo				
	Mean	Median	Minimum	Maximum	Mean	Median	Minimum	Maximum	
Kt/v									\
età	52.89	53.00	28	77					\
anni trapianto	6.67	4.00	1	30	10.56	12.00	1	22	0.118
Creatiniemia	1.67	1.50	1	4	2.52	1.40	1	9	0.945
Proteinuria	37.59	0.00	0	412	48.00	0.00	0	300	0.885
Hb	12.49	12.40	8	16	12.60	12.40	11	15	0.994
AST	16.87	16.00	6	53	14.25	14.50	9	18	0.278
ALT	14.91	12.50	4	53	11.25	10.00	6	26	0.1
VES	19.95	15.00	2	63	30.40	34.00	8	51	0.172
PCR	15.41	.17		960	.14	.09			0.451
GB	6.43	6.04	2	13	7.54	7.29	6	11	0.043
N	3.79	3.54	1	9	4.75	4.73	3	7	0.095
L	1.80	1.68		4	1.87	1.47	1	4	0.825
M	.48	.47		1	.48	.49		1	0.889
E	152.81	.10		9.504	.17	.18			0.394

Tab . 5 Comparisons selecting only the transplanted

GB : medians differ statistically significantly (6:04 vs. 7:29)

Using to compare the renal transplant patients in relation to positive or not the test for simkania has shown that there is a positive correlation between simkania and white blood cell count , reaching statistical significance (p 0.043) .

SOLO DIALIZZATI	Simkania								Kruskal-Wallis, p-value
	Negativo				Positivo				
	Mean	Median	Minimum	Maximum	Mean	Median	Minimum	Maximum	
Kt/v	63.80	66.05	23	80	66.83	66.15	59	74	0.448
età	68.48	71.50	19	90					\
anni trapianto									\
Creatiniemia	8.58	8.80	3	15	7.26	7.70	4	11	0.137
Proteinuria									\
Hb	868.62	10.15	8	36.060	10.18	9.85	8	13	0.731
AST	17.37	12.00	4	140	13.00	11.00	6	29	0.318
ALT	14.05	12.00	3	64	12.45	9.00	2	33	0.458
VES	40.28	37.50	2	84	48.90	47.00	25	101	0.311
PCR	134.87	.67		3.660	1.60	.78		6	0.79
GB	6.31	6.18	2	14	8.28	6.94	3	23	0.284
N	3.57	3.30	1	9	5.25	4.26	2	15	0.068
L	1.56	1.17		4	1.51	1.50		3	0.772
M	.44	.45		1	.43	.43		1	0.693
E	.25	.16		1	.28	.19		1	0.429

Tab . 6 Comparisons selecting only the dialysis

Comparing patients on hemodialysis in relation to test positive for simkania did not show any statistically significant difference .

The statistical analysis can not be carried out on the IgA IgG S. Negevensis as too reduced the sample size .

Discussion and Conclusions

Simkania negevensis resists destruction by free-living amoebae and that may use the amoebae as an environmental reservoir (). Its role as an emerging human respiratory pathogen is suspected (). Epidemiological studies using PCR, cell culture, and serology have documented not only the worldwide presence of this microorganism but also its association with bronchiolitis in infants and with lower respiratory tract infections in adults. More recently, *S. negevensis* DNA has also been amplified from human arterial biopsy specimens.

In our study S. negevensis was tested and found positive in the samples of water taken from the circuits for hemodialysis , which is carried out from power the preparation of water for dialysis , before and after disinfection treatments , resulting always positive. Our study identifies a population that is unexpected positive the SN , the hemodialysis patient. This is to be interpreted in the context of the life of the bacterium . Like other Chlamydia-related organisms, S. negevensis may infect free-living amoebae such as Acanthamoeba which are widespread in water, including hospital water supplies (La Scola et al., 2002). It spreads in the waters of the network circuits for dialysis, even after the treatments to which are subjected the waters .

Hospitalized patients exposed to aerosolized particles, such as those undergoing mechanical ventilation, may thus be colonized or infected by these intracellular bacteria, whose pathogenic role in this setting has been poorly investigated.

However, it is worth noting that in vitro studies of the pathogenesis of S. negevensis have demonstrated its ability to infect human macrophages and to induce a host cell inflammatory response, supporting its potential ability to cause human infections and the need for further clinical investigations (Kahane et al., 2008).

Free-living amoebae represent a widespread evolutionary niche that may favour the selection of virulence traits in intra-amoebal bacteria, enabling them to survive in other phagocytic cells, including alveolar macrophages, which are one of a major line of immune defence against invading pathogens. The examples of emerging pathogens provided probably represent only the tip of the iceberg, and there is still a largely underestimated biodiversity of amoebae-resisting bacteria, which may have acquired their ability to cause diseases in humans by the development of virulence traits

during their intra-amoebal life. This huge and so far unexplored biodiversity not only includes members of the clade presented , i.e. Legionella spp., Chlamydiae, Bradyrhizobiaceae and mycobacteria, but also many other bacterial clades and giant viruses (Greub & Raoult, 2004).

From a recent study (Donati et al 2013) S. negevensis presents , despite the lack of reference literature , a prevalence comparable to that of the general population or in patients on dialysis (28.9 %) that transplanted (15.5 %) . The prevalence of IgA is elevated in dialysis patients , while being comparable to that of the general population (in transplant patients). In our work, it was not possible to collect data on the IgA IgG vs SN because the sample was small in numbers .

S. negevensis has a specific behavior towards patients transplanted , recovering levels of seroprevalence overlapping with the general population ; in contrast to what happens for the C. pneumoniae with which it shares several characteristics .

This could be explained by the versatility already highlighted in vitro, of the s . negevensis , inducing different types of infection (active , persistent , cryptic) , the whose characteristics in vivo, however, are yet to be determined for both the healthy population and for the immunodepressed .

Solid organ transplantation is often the only therapeutic option for end-stage organ failure, but lifelong immunosuppression is necessary to prevent the rejection of allogeneic transplants. High frequencies of alloreactive T cells contribute to the vigour of the allogeneic response that mediates rejection. These alloreactive T cells recognize either allogeneic MHC molecules (a process termed direct allorecognition) or peptides from donor MHC molecules or minor histocompatibility antigens presented on recipient antigen-presenting cells (APCs) (a process termed indirect allorecognition).

Observations from experimental models indicate that events associated with clinical transplantation, such as ischaemia–reperfusion injury and infections, can enhance alloreactivity and promote rejection episodes through the release of damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs), as well as through alterations in the alloreactive T cell repertoire.

Despite the observed correlation between increased frequencies of infections and acute rejection, especially in the early post-transplant period, definitive clinical proof that infections stimulate alloreactivity is lacking. It was discussed the difficulty in making this causal link: immunosuppression used to prevent or treat transplant rejection episodes increases the susceptibility

to developing infections but also reduces some of the pro-inflammatory responses normally elicited by the infections. Furthermore, some infections trigger the release of endogenous corticosteroids that can compromise the pro- inflammatory response to infection. The prophylactic use of antibiotics, antifungal drugs and antiviral drugs can also reduce the impact of infections on alloreactivity. Finally, tapering of immunosuppression to treat some types of viral infection may increase the likelihood of rejection, which then makes a definitive demonstration of the direct participation of infections in transplant rejection even more challenging. Infections that occur before transplantation include those implicated in the cause of organ failure, such as hepatitis B virus (HBV) or HCV infections in the case of liver damage, and those associated with end-stage dis- ease, such as bacterial and fungal infections in patients with cystic fibrosis and in patients with end-stage renal failure on haemodialysis or peritoneal dialysis. Infections in the post-transplant period are shaped by the type of immunosuppression regimen used, the presence of infections in the donor organ or recipient, and the use of prophylactic antimicrobial agents.

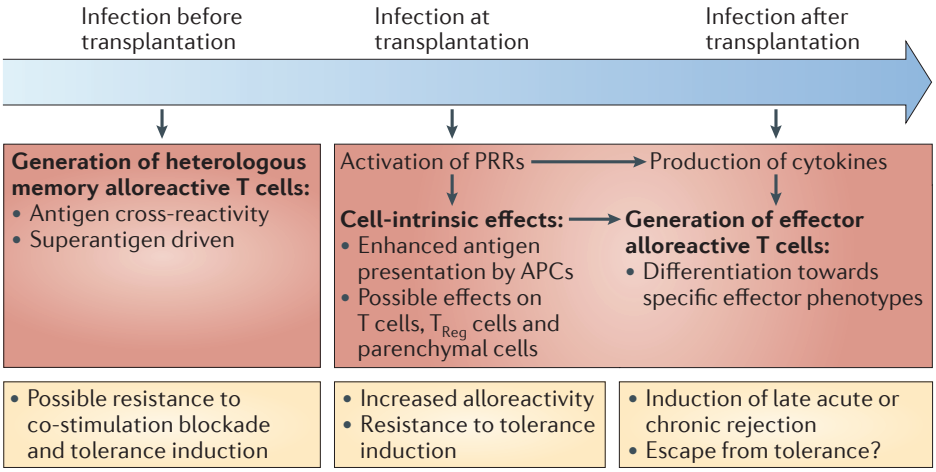


Figure 1 | **Possible effects of infections before, at and after transplantation.** Some T cells specific for microbial peptides presented by self MHC molecules can cross-react with allogeneic MHC molecules, and bacterial superantigens can directly activate large populations of T cells. Therefore, infections experienced before transplantation can give rise to heterologous memory alloreactive T cells that may be more resistant to immunosuppression than naive T cells[15,18]. The engagement of pattern-recognition receptors (PRRs) on antigen-presenting cells (APCs), T cells and/or parenchymal cells at the time of or after transplantation can induce various signals and cytokines. This can result in enhanced priming, survival and expansion of alloreactive T cells, as well as dictating the phenotype of differentiating alloreactive T cells. Infections occurring late after transplantation may elicit pro-inflammatory signals that activate tolerant T cells by enabling their escape from immunosuppression and/or peripheral mechanisms of tolerance, thereby precipitating rejection. T_{Reg} cell, regulatory T cell.

Pathogenic bacterial, fungal and viral infections are prevalent in the peri- and post-transplant periods as a result of surgical insult and immunosuppression. Studies evaluating defined categories of bacterial infections in transplant recipients have been able to link the incidence of bacterial infections with transplant rejection. In patients who have received lung transplants, infections with *Chlamydia pneumoniae* or *Simkania negevensis*, and colonization of the allograft by *Pseudomonas aeruginosa* or *Aspergillus* spp., have been associated with the development of acute rejection or bronchiolitis obliterans syndrome. Furthermore, urinary tract infections (especially those occurring late after renal transplantation when prophylactic antibiotics have been discontinued) have been associated with chronic rejection and renal graft loss. When infections occur locally in the transplanted organ, graft injury can result from immune responses directed at the pathogen, from the reactivation of alloreactivity and/or from the activation of T cells that recognize cryptic autoantigens exposed following cell damage. Despite these reports, unambiguous clinical evidence for a causal relationship between infection and transplant rejection is currently lacking.

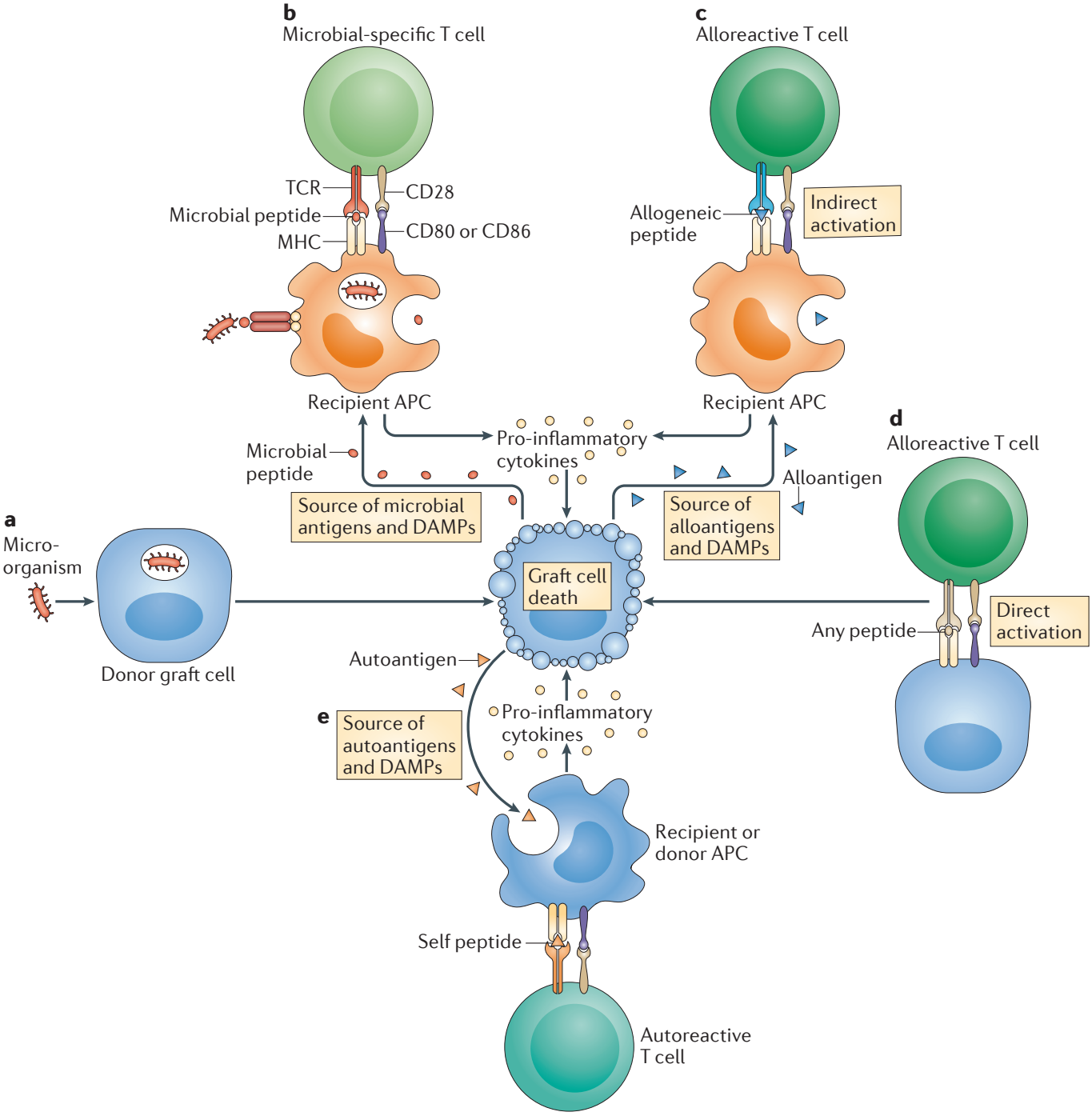


fig 5 potential causes of graft damage during microbial infections. graft destruction during an infection can occur through multiple mechanisms ***a****. microorganisms with a tropism for the graft organ can have direct cytopathic effects that cause the release of Damage-associated molecular patterns (DAMPs) as well as microbial or donor antigens (alloantigens) and possibly autoantigens.* ***b*** *recipient antigen-presenting cells (APCs) or endothelial cells in the graft take up and cross-*

present exogenous microbial antigens to recipient T cells. Activated APCs and or T cells secrete cytokines that are damaging to the graft or that injure endothelial cells, thereby reducing vascular supply to the graft. ***c*** *infections can induce the maturation of recipient APCs or activate endothelial cells that have taken up donor antigens (blue triangles). This results in the indirect activation of alloreactive T cells and graft injury mediated by pro inflammatory cytokines and toxic soluble factors that injure blood vessels.* ***d****. infections can augment the direct activation of alloreactive T cells by enhancing the maturation of donor APCs or graft parenchymal cells, resulting in graft damage.****e*** *cellular graft damage may release cryptic antigen (autoantigen) and DAMPs that then activate auto reactive T cells*

The complex effects of infections and tissue damage on the outcome of transplantation are starting to be appre- ciated. Not only do such events induce the generation of memory alloreactive T and possibly B cells, but they also give rise to PAMPs and DAMPs, which can promote innate immune responses. The tools to analyse the com- plex feedback circuits between immune responses and the host microbiota (BOX 3) or infections are just being developed, with some pathways known and some postulated to have an impact on alloreactivity. It is likely that further study of how these circuits impinge on allo- responses will continue to change our understanding of organ rejection and tolerance, and will provide insights into how the effects of infection on alloreactivity can be controlled without compromising protective immunity(62).

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