An overview of *Rickettsia* diagnostics relevant for Dutch travellers

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Rickettsioses are zoonotic infections and are emerging infectious diseases among international travellers. Over 450 travel-associated cases have been reported worldwide yet much remains unknown about the scope of these infections. With an increase in recent decades of travel to the tropics, ecotourism and backpacking, it is expected these infections will be continually and increasingly seen in the future. Diagnosis of infections is notoriously difficult, with many methods available however none optimal at confirming diagnosis. Molecular methods are recommended for diagnosis in the early acute phase of rickettsial infections. Using polymerase chain reaction early infection can be identified in patients within days of inoculation. Serology can only be used for diagnosis in the late acute phase of infection, once IgM and IgG levels are detectable. Immunofluorescence assays act as the gold standard for serology, and provide a quick and reliable means of confirming rickettsial infection. Culturing should only be undertaken in severe cases where species diagnosis is of utmost importance. Rickettsioses should always be considered as a differential diagnosis for patients presenting with febrile illness and a suspect travel history.

Introduction

Rickettsioses are a group of zoonotic infections transmitted by vectors such as ticks or mites from various animal host reservoirs to humans. It is an emerging infectious disease, notably amongst international travellers. The *Rickettsia* bacterium is a small, obligate, intracellular Gram-negative coccobacilli (Jensenius et al. 2004a; Cowan et al. 2009). The Rickettsiales is an order consisting of four different genera: *Rickettsia, Orientia, Ehrlichia* and *Anaplasma* (Jensenius et al. 2009). The genera *Rickettsia* and *Orientia* together cause rickettsioses and will be focussed on for the purpose of this review. The current taxonomy of Rickettsiales is intricate, with new species being identified and others being updated constantly. Currently it is thought that there are over 12 different species of *Rickettsia*, which are subdivided into the spotted fever group comprised of 10+ species and typhus group consisting of 2 species (Jensenius et al 2004a). Within the *Orientia* genera there is currently only one species, namely *O. tsutsugamushi* (Jensenius et al. 2004a, 2009).

Acute rickettsiosis generally presents with non-specific flu-like symptoms such as fever, either or not accompanied by other symptoms such as headaches and myalgia (Cowan et al. 2009). More characteristic symptoms are an inoculation eschar and a rash, however, these are not always observed (Jensenius et al. 2004a). Rickettsioses are usually self-limiting diseases however, in certain cases clinical manifestations are more severe and may cause mortality if left untreated. Due to the worldwide spread of the different subspecies of these bacteria, knowledge of the different diseases is of

utmost importance for the clinician. However, due to the non-specific clinical manifestations of rickettsial disease the diagnosis is often missed, delaying treatment with consequent progression into severe forms (Cowan et al. 2009). The challenge with these diseases lies therefore in the timely recognition of infection.

Treatment of rickettsial infection is fairly straightforward. Usually tetracyclines, preferably doxycycline, are prescribed as a daily dose either orally or intravenously for 7 to 10 days. Chloramphenicol can also be given in 6-hourly doses orally or intravenously for 7 days and is the best drug to use in case of pregnancy (Cowan et al. 2009; Jensenius et al. 2004a). If a patient is nauseous or vomiting, the intravenous route should always be used for drug administration. Alternatively, fluoroquinolones can be administered in cases where tetracyclines cannot be used such as children. However, alternatives are generally less effective than doxycycline (Cowan et al. 2009).

Currently over 450 different cases of "travel-associated rickettsioses" have been described in literature worldwide, with most being attributed to *Rickettsia typhi, Rickettsia africae,* and *Orientia tsutsugamushi* (Jensenius et al. 2004a). In a 2007 study by Wilson and co-workers among 7,000 travellers presenting with fever, 2% was attributed to rickettsioses and of these 20% were hospitalised (Wilson et al., 2007). Under diagnosis is assumed to be widespread due to a lack of knowledge about symptoms, etiology and mechanisms of the disease and the fact that most case studies included only small cohorts (Jensenius et al. 2009).

Approximately 50 million Western travellers visit tropical areas each year (Jensenius et al. 2013; Antinori et al. 2004). Of these an increasing amount visits remote places where trekking, safaris and camping are central activities. Reflecting the world's increasing propensity for ecotourism, these activities are increasingly undertaken in wilder and more overgrown areas in the tropics (Jensenius et al. 2009, Jensenius et al. 2006). Due to the wide spread of rickettsial diseases in the world, and the fact that most vectors are found in the tropics, an increase in travel-associated rickettsioses should be anticipated for the coming decades. With a yearly increase in adventure travel of 10% since 1985, this expectation is further supported (Jensenius et al. 2006). Furthermore, as ticks and mites prefer warmer conditions, an increase in temperature due to climate change could cause rickettsioses to spread further into Europe (Parola, 2004). Around 3-11% of travellers report fever-like symptoms upon return to their home country (Antinori et al. 2004). It was reported that 13.7% of all travellers returning from southern Africa who visited their health care professional due to illness were diagnosed with one of the spotted fever rickettsioses (Jensenius et al. 2009).

According to the Centraal Bureau voor de Statistiek (CBS) in 2012 over 18 million Dutch tourists travelled abroad (see Table 1). Many had destinations endemic for disease vectors such as France (15%), Portugal (2.4%) and Turkey (4.4%). Mediterranean spotted fever is endemic in these three countries, albeit that behavioural factors during holiday will only bring a small proportion of tourists into contact with ticks or mites. Also there seems to be an increase in young backpackers from the Netherlands, who prefer staying in more rural areas and exploring the wilderness of the countries they visit. Though the diagnosis of rickettsial infections in international travellers is rare, the amount and locations of holidays taken by the Dutch does mean that they are at risk of contracting these zoonoses. The four most

commonly found rickettsioses among Dutch travellers would be *R. africae, R. conorii, R. typhi* and *O. tsutsugamushi*. This prediction is based on cross-referencing the regions of vector endemicity and the regions where travellers from the Netherlands go on holiday. A short description of the four relevant rickettsioses will be given below.

This review will focus on the diagnostic tools that can be used to identify four main rickettsioses found amongst international travellers that are prevalent in countries where Dutch tourists tend to travel.

Country	Holidays taken abroad by Dutch travellers	
	Number of holidays (x1000)	Percentage (%)
Belgium	1,811	9.7
Luxembourg	178	1.0
France	2,798	15.0
Spain	1,796	9.6
Portugal	439	2.4
Austria	1,233	6.6
Switzerland	286	1.5
United Kingdom	771	4.1
Norway, Sweden, Finland	320	1.7
Denmark	200	1.1
Germany	3,400	18.3
Italy	1,030	5.5
Greece	645	3.5
Hungary	159	0.9
Czech Republic	209	1.1
Turkey	814	4.4
Egypt	247	1.3
Asia	306	1.6
United States of America	463	2.5
Caribbean	87	0.5
Other	1,438	7.7
Total	18,628	100

Table 1. Holidays taken by Dutch travellers in 2012 distinguished by country or region of travel. Data acquired from Centraal Bureau voor de Statistiek.

Methods

Papers were collected using PubMed. Initially a general approach was used, to gain some insight into the amount and type of papers available. Specific MeSH terms were used for the literature search: “Rickettsia”, “Rickettsieae” and “Rickettsiaceae”. Using just the MeSH terms returned over 4,500 papers therefore a more precise method was needed. Additionally adding “diagnosis” to the MeSH terms returned 1,753 papers. Searches were therefore further concentrated using other specific terms or words to be able to focus our search on international travellers and the Netherlands.

To do this a series of different searches were carried out, as most papers are specific in a topic of *Rickettsia* or to a country. ““Rickettsia"[Mesh] OR "Rickettsieae"[Mesh] AND the Netherlands” was used as an initial focused search term, which returned 15 papers. Specific keywords that were then added to the MeSH terms were; diagnostics, international, travel, Europe, human, epidemiology, France, serology, IFA, rapid test, molecular, culture, africae, conorii, typhi, tsutsugamushi, and rapid diagnostic test. These searches were used to define varying topics that are part of the review. A small amount of papers were found with each search, for example the MeSH terms combined with “diagnostics” only returned 11 papers. The final selection of papers therefore came from the many different individual searches that were done. A total of 74 papers were selected and read for this literature review and 40 papers were used. When papers were not freely available via PubMed, the ScienceDirect database was used to gain access to most if not all papers.

Rickettsial Diseases

Rickettsia africae

Etiology: African tick bite fever is currently the most commonly reported rickettsiosis in international travellers (Jensenius et al. 2004a). It is caused by the bacteria *Rickettsia africae*. The vectors of *R. africae* are cattle ticks belonging to the *Amblyomma* genus. The main vectors are the species *Amblyomma variegatum* and *Amblyomma hebraeum*, each endemic to different regions where *R. africae* is encountered (Althaus et al. 2010; Jensenius et al. 2004b).

A. variegatum (Source: CDC)

Distribution: *R. africae* is prevalent in sub-Saharan Africa (see Figure 1) (Althaus et al. 2010; Jensenius et al. 2004a; Jensenius et al. 2004b). Travellers usually come into contact with the agent in rural areas, often during safaris or bush walks. Most infections have been encountered in South Africa, Zimbabwe and Botswana (Jensenius, 2004a; Jensenius et al. 2003). The ticks are commonly found on vegetation and are known to have an aggressive hunting strategy, actively seeking out their hosts. Cases are therefore often seen in a clustered manner with many people in a group presenting with the disease and often with multiple eschars (see Figure 2) (Althaus et al. 2010; Jensenius et al. 2004b; Fournier et al. 2002). Prevalence among travellers is high, with *R. africae* being the second most common disease brought back from Africa after malaria. Incidence rates have been estimated between 4% and 5.3% (Jensenius et al. 2003).

Signs and symptoms: The disease is usually mild and self-limiting. Clinical symptoms vary between patients and may include headaches, neck myalgia, inoculation eschars and fever (Althaus et al. 2010; Jensenius et al. 2003). In 20-30% of the cases a cutaneous rash is observed (Althaus et al. 2010). The incubation period of the disease is 5-10 days, and differential diagnoses are often made of malaria or typhoid fever (Cowan et al. 2009; Reshef et al. 2007).

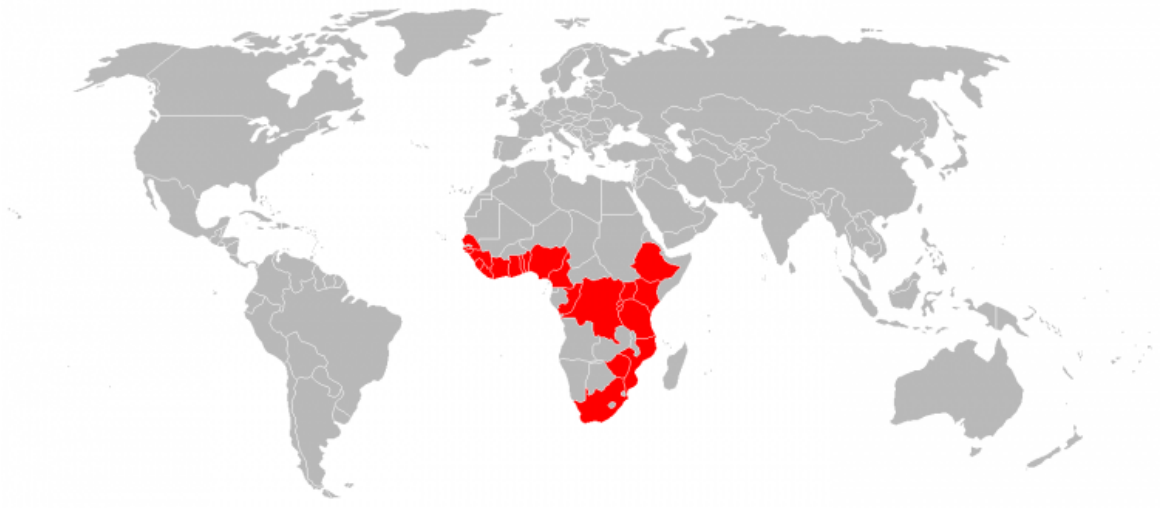

Figure 1. Map showing distribution of African tick bite fever. Countries at risk were defined using data from Althaus et al. 2010; Jensenius et al. 2004a; Jensenius et al. 2004b.

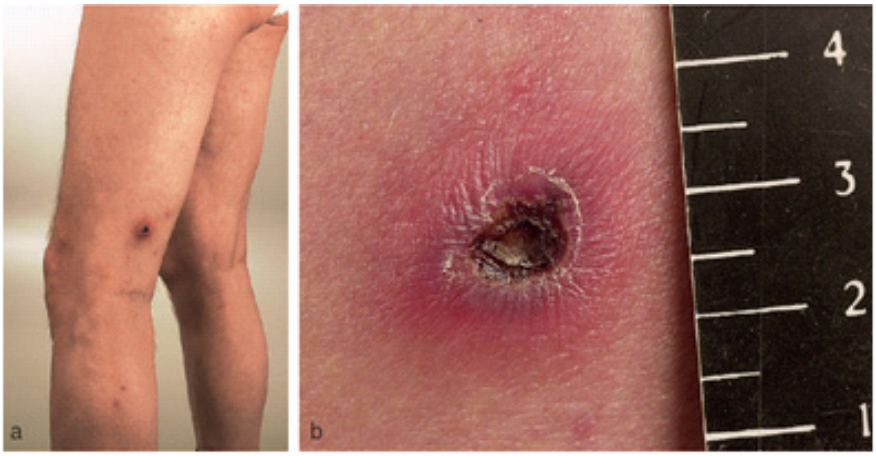

Figure 2. Inoculation eschar on African tick bite fever patient. Source: Diederen et al. 2003.

Rickettsia conorii

Etiology: Mediterranean spotted fever, also known as fièvre boutonneuse, is caused by the *Rickettsia conorii* bacteria. The bacteria are transmitted to humans by dog ticks, most commonly the *Rhipicephalus sanguineus* tick (Cowan et al. 2009). The ticks are mostly host specific and do not readily feed on humans unless dogs are unavailable (Brouqui, 2007). *R. conorii* is subdivided into four different subspecies; *conorii*, *indica*, *israelensis* and *caspiae*, all endemic to

R. sanguineus (Source: CDC)

different regions of the world, however, our focus will be on *R. conorii conorii* as it is most commonly encountered (Wood 2012).

Distribution: *R. conorii* is endemic to the Mediterannean, but is also found in regions of Africa and Asia (see Figure 3) (Cowan et al. 2009; Jensenius et al. 2004a; Jensenius et al. 2004b). Incidence is highest in Portugal, with 9.8 infected cases per 100,000 people, and cases are continuously identified in new countries, the most recent being Turkey (Parola, 2005).

Signs and symptoms: Symptoms are flu-like with patients presenting with acute fever and muscle pains and in 70% of patients an inoculation eschar is seen (Oteo, 2012; Jensenius et al. 2004b). Maculopapular rash is extremely common in Mediterranean spotted fever; over 95% of patients present with this rash on their extremities (Oteo; 2012; Tonna, 2006). Recovery of the disease is slow, and many patients experience complications including neurological problems and multiorgan failure (5-6% of cases), with a case fatality rate (CFR) of 2% (Oteo; 2012; Parola, 2005; Jensenius et al. 2004b).

Treatment anomalies: Treatment with fluoroquinolones can worsen the disease course (Oteo, 2012).

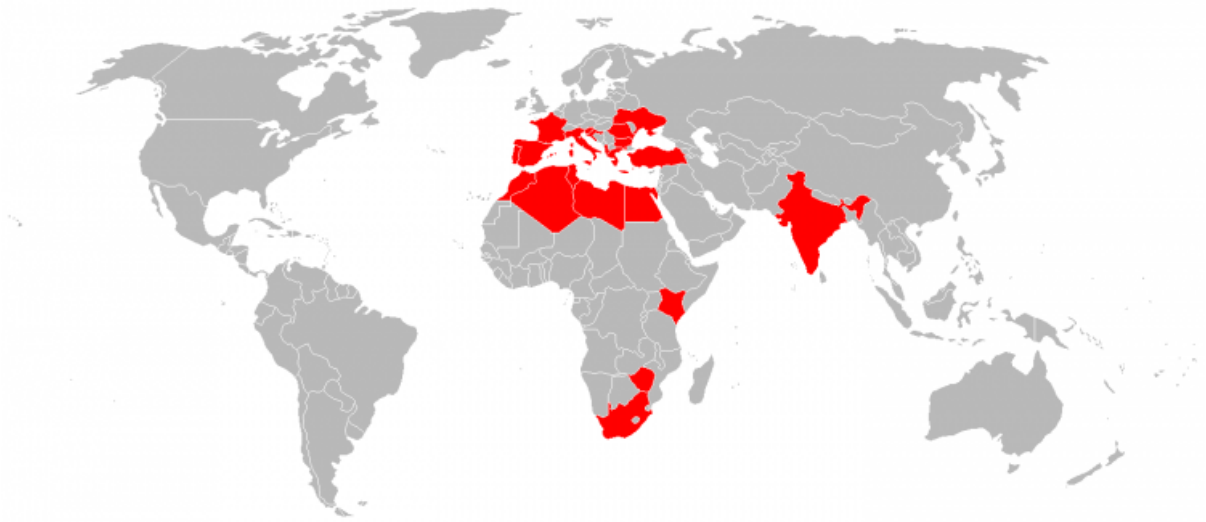

Figure 3. Map showing distribution of Mediterranean spotted fever. Countries at risk were defined using data from Cowan et al. 2009; Jensenius et al. 2004a; Jensenius et al. 2004b.

Rickettsia typhi

Etiology: Murine typhus, caused by *Rickettsia typhi*, is a rickettsial disease that belongs to the typhus subgroup (Walter, 2012; Jensenius et al. 2004a). Several rodent species, predominantly rats, are the main reservoir. The rat flea, *Xenopsylla cheopsis,* is the vector (Parola, 1998). The main route of infection is through fleabites, where the fleas leave infective faeces behind in the bite or wound (Walter, 2012; Jensenius et al. 2004a).

X. cheopsis (Source: CDC)

Distribution: The disease is spread worldwide with regions in Asia, Africa, the Americas and Europe being affected. Most cases occur near portal cities or beaches where rats are abundant (see Figure 4).

Signs and symptoms: The disease is often not recognized or misdiagnosed. Symptoms are almost exclusively non-specific. Eschars are not present and rashes and other complications are uncommon. The CFR is 1-2% (Cowan et al. 2009; Jensenius et al. 2004a; Pether et al. 1994).

Treatment anomalies: Doxycycline is the drug of choice and extensive delousing should occur for all suspected patients (Cowan et al. 2009).

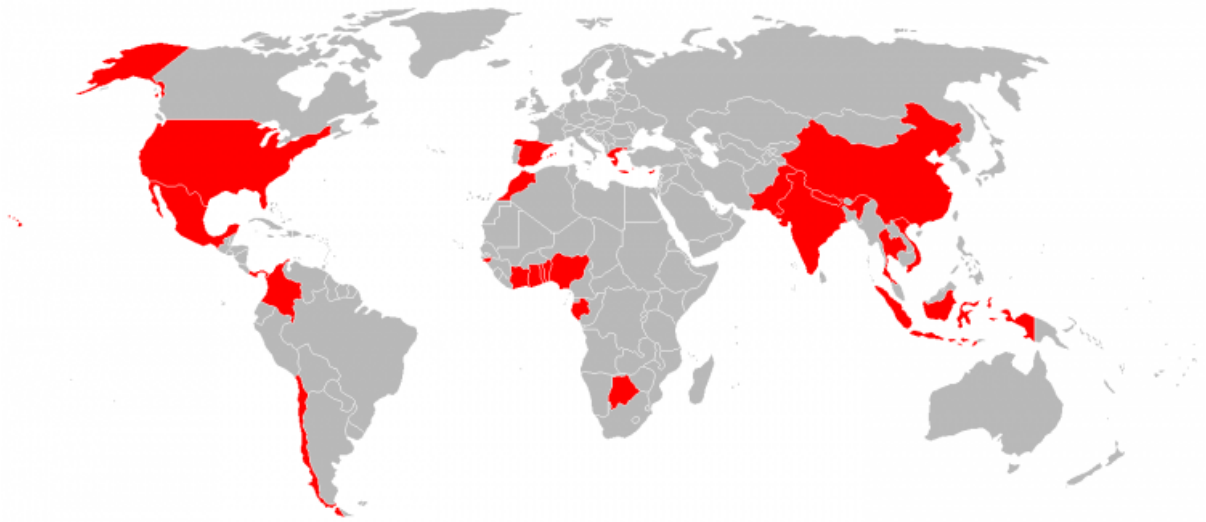

Figure 4. Map showing distribution of murine typhus. Countries at risk were defined using data from Walter, 2012; Jensenius et al. 2004a.

Orientia tsutsugamushi

Etiology: Scrub typhus is caused by *Orientia tsutsugamushi* bacteria. It is transmitted to humans by larval trombiculid mites, also known as chiggers, which are also thought to be the main reservoir. Their activity depends on humidity and temperature (Watt and Parola 2010).

Chigger (Source: www.chigarid.com)

Distribution: The zoonosis is found in the Asia-Pacific region (see Figure 5) (Blacksell et al. 2010; Watt and Parola, 2003). In this region the disease is commonly observed with an estimated number of cases at 1 million a year for the local population (Jensenius et al. 2004a).

Signs and symptoms: Bites typically occur in the genital region or on the lower extremities, and in 50-80% of cases an eschar can be observed. Symptoms of scrub typhus include flu-like symptoms. Lymphadenopathy with regional swelling of the lymph nodes is characteristic but not pathognomic for scrub typhus (Watt and Parola,

2003). Infection with *O. tsutsugamushi* can include complications such as adult respiratory distress syndrome and sceptic shock. Multi-organ failure has also been observed in two travellers to Thailand in the past (Cowan et al. 2009; Jensenius et al. 2004a). Pre-emptive treatment is therefore of utmost importance (Watt and Parola 2003). Arbovirus infections, malaria and leptospirosis are often diagnosed instead of scrub typhus (Cowan et al. 2009).

Treatment anomalies: In certain regions, including northern Thailand, *O. tsutsugamushi* has developed antibiotic resistance to certain drugs such as chloramphenicol (Cowan et al. 2009).

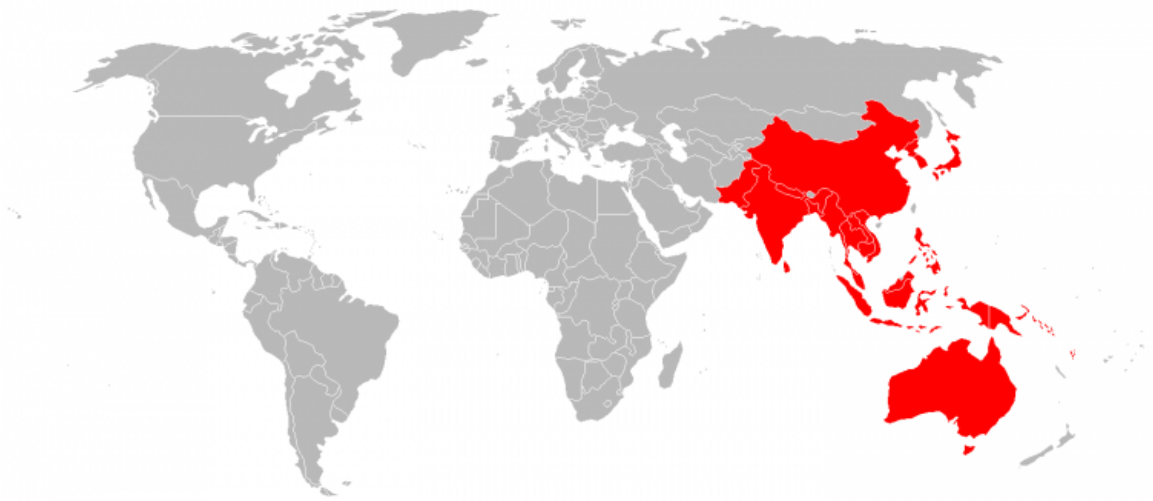

Figure 5. Map showing distribution of scrub typhus in the Asia-Pacific. Countries at risk were defined using data from Blacksell et al. 2010; Jensenius et al. 2004a; Watt and Parola, 2003.

Diagnostic testing

Rickettsial diseases are notoriously difficult to diagnose. Several methods are available yet almost none is optimal at confirming the diagnosis. The three main methods of diagnosing rickettsial disease in patients are through serology, molecular techniques or culture. Certain rapid diagnostic tests are also available but lack diagnostic accuracy in terms of sensitivity and/or specificity to be utilised commercially. All methods will be explained here. Differences between the *Rickettsia* and *Orientia* strains are highlighted, if applicable.

Samples for diagnosis

For definite diagnosis certain tissue samples are useful specimens. As the site of entry and because of local multiplication of *Rickettsias*, eschars contain high concentrations of bacteria. They therefore present optimal samples for diagnosis by culturing or molecular detection (Lepidi, 2006). Sampling by using an eschar swab is an easy, non-invasive method that can even be done before flu-like symptoms are present. A piece of eschar crust from the scab can also be used and has proven to be quick and painlessly obtainable from the patient. This enables application in rural settings as well (Socolovschi, 2012). However, due to the lesion not being painful, eschars often go unnoticed (Richards, 2012). Alternatively a skin biopsy can be taken from the rash where high concentrations of the bacterium are also present (Richards, 2012). Skin biopsies have a number of drawbacks. It is an invasive method that can have side effects. Many patients reject having a skin biopsy taken and prefer collection of blood (Richards, 2012; Mouffok, 2011). Furthermore, the rash in patients is often not present and usually appears late after presentation of the disease (± 5 days). If an eschar is not present, however, a rash might present a good alternative for efficient sampling.

Blood samples, serum or plasma can also be used. When taking these samples a few crucial things should be considered. Firstly, unless it is an extreme infection, the level of bacteria in the blood is less than in tissue samples from rash or eschar, and therefore holds an increased risk for false negative results (Richards, 2012; Wood, 2012). Furthermore, the levels of antibodies in the blood may not be apparent yet at an early time of blood collection and hence serology does not present a valuable approach for early diagnosis (discussed further later) (Richards, 2012; Althaus et al. 2010; Jensenius et al. 2004b). It should be noted that, whatever the sample, antibiotic treatment prior to sampling reduces the efficacy of diagnosis either by culture, serology or molecular methods (Angelakis et al. 2012).

Each method of diagnosing rickettsial infections has its own advantages and drawbacks. A summary of when markers can be taken and when methods can be utilised can be seen in Figure 6. This graph illustrates that culturing and molecular methods can be used for diagnosis in the early acute phase of the infection. Serology can only be used as a diagnostic method once IgM and IgG become detectable, which is not until around 10 days post-inoculation. The antibodies become detectable around the same time however higher levels of IgM are noted in an earlier phase, whilst IgG levels remain high for a longer duration. Many conclude that a combination of both serology and molecular methods is the most successful way of diagnosing infection

and identifying which strain of *Rickettsia* is present in a patient (Richards, 2012; Wood, 2012).

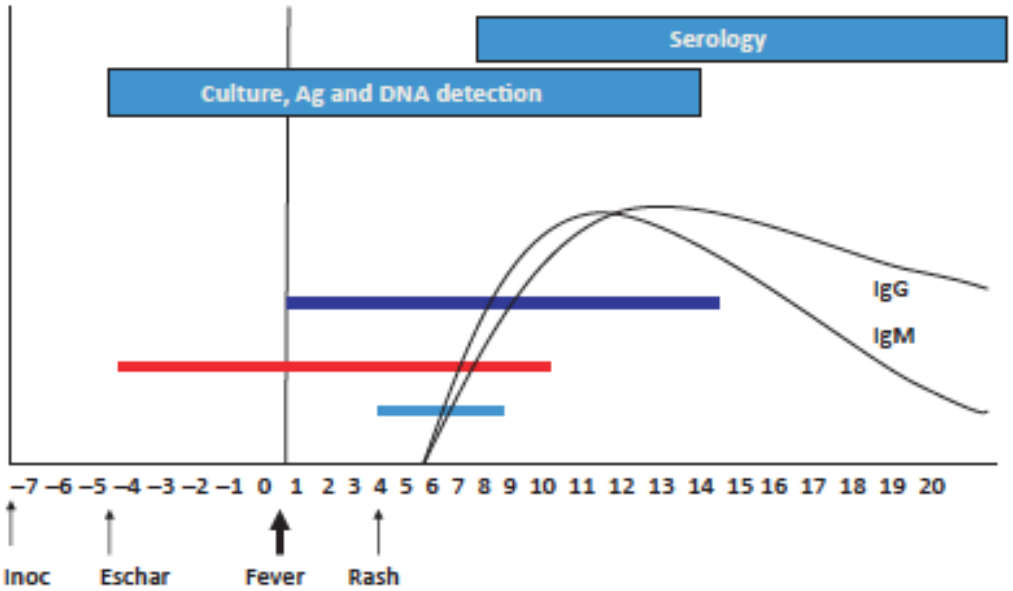

Figure 6. Time course of rickettsial disease markers and when certain diagnostic tools can be used. Image taken from Richards, 2012. "Inoc" serves as the starting point where the host patient is infected by the vector. Fever is then encountered within 5 to 10 days after inoculation after which the rash, if present, may occur at a later stage. IgM and IgG levels can be seen appearing around 10 days after eschar formation, and once levels become detectable serology can be done. Culturing can be done from the moment an eschar is found. This is a general image, showing the standard case for most *Rickettsia* strains, however in the case of *Rickettsia africae* IgM and IgG levels are only detectable after 25-28 days.

Diagnosis by culturing

Culturing the rickettsia bacteria provides proof of infections and as such presents the gold standard for diagnosis (Richards, 2012; Jensenius, et al. 2004b). A culture can become positive within 48-72 hours after inoculation of the culture medium and thus provides a confirmative diagnosis at the early acute stage of the disease when serology is still insensitive. Culturing is done using the shell-vial cell culture assay and is critical in further describing *Rickettsia* species genetically and physiologically (Angelakis et al. 2012). The shell-vial culture technique was developed to allow for the isolation of fastidious organisms and uses human embryonic lung fibroblasts or L929 mouse fibroblasts for culturing of *Rickettsia* spp. (Gouriet et al. 2005). The samples that can be used for this technique include skin biopsies, heparinised blood, or hemolymph from the vector. Culturing is done in Eagle's minimal essential medium (MEM) supplemented with 10% FCS and 2mM L-glutamine/liter. Detection can usually be done 7 days later using Gimenez or immunofluorescence staining (Gouriet et al. 2005).

Culturing is, however, restricted to reference laboratories because it is a potentially hazardous and technically demanding method that must be performed at a biosafety level 3 (Angelakis et al. 2012; Gouriet et al. 2005). Furthermore, culturing is not very sensitive and prone to failure in case antibiotic treatment has already been started

(Richards, 2012; Angelakis et al. 2012; Brouqui, 2004). Due to the difficult nature of culturing and the need for it to be performed in a specialised laboratory, it is often recommended to only use culturing in cases where the disease is so severe that species diagnosis is of utmost importance (Jensenius et al. 2004a).

Diagnosis by serology

Serology is the most commonly used diagnostic approach for rickettsial disease throughout the world (Brouqui, 2004; Jensenius et al. 2004b). It allows for the detection of antibodies against *Rickettsia* in serum or plasma samples. Immunofluorescence assays (IFA) are the references tests, also denoted as gold standard, in detecting rickettsial disease through IgG or IgM levels (Premaratna, 2012). Other serological methods include the Weil-Felix assay, enzyme-linked immunosorbent assay (ELISA) and Western blot analysis (Richards, 2012; Althaus et al. 2010). The downside of serology is that available serological tests (see below) are not sufficiently specific to detect the individual species of *Rickettsia*. This is due to the cross-reactivity among the spotted fever rickettsioses group (Brouqui et al. 2004). Since various distinct rickettsial species are present in several countries, serology is not very useful for epidemiological purposes (Richards, 2012; Althaus et al. 2010; Jensenius, et al. 2004b). As indicated before, serology is not sensitive in the early acute phase of the disease when most patients tend to seek medical aid (Jensenius et al. 2009).

i. Immunofluorescence Assay (IFA). IFA is a technique that identifies antibodies bound to specific antigens through the use of fluorescent dyes. Usually antibody levels are detectable around 5 to 10 days following fever, however, for African tick bite fever patients seroconversion happens later (Richards, 2012). For example the average time of seroconversion for Mediterranean spotted fever patients for IgG and IgM are 6 and 9 days respectively, whilst for African tick bite fever patients this is 28 days for IgG and 25 days for IgM (Beltrame, 2012). This highlights the main problem that is encountered in IFA; that detection of IgG and IgM levels differs between species. Occasionally a delayed reaction occurs and differences can be observed between patients as well. This indicates that diagnosis on a single acute serum sample used for serology may not be possible or reliable. It is therefore of utmost importance to obtain both an acute and convalescent phase sample of blood, at least two weeks apart, in order to be able to compare results and make a definite diagnosis (Richards, 2012; Beltrame et al. 2012; Jensenius et al. 2004a). Studies by both Beltrame et al. (2012) and Fournier et al. (2002) have also shown that in the case of African tick bite fever seroconversion may not occur when patients have been given doxycycline in early disease stages.

ii. Western blotting. In reference laboratories, once rickettsial disease has been confirmed using IFA, Western blot analysis can identify the species through cross-adsorption (Richards, 2012). Moreover, IFA and Western blotting are complimentary. A study by Fournier et al. (2002) indicated that only 15% of patients could be diagnosed to species level using solely IFA, whilst in the combination with Western blotting 73% of patients were identified as being infected with *R. africae.* On the other hand, Western blotting on its own gives many false positive results due to the cross-reaction between antibodies and

therefore it is only recommended in conjunction with IFA (Brouqui et al. 2004).

iii. Weil-Felix assay. The most easily used, cheap and widely available serological method to test for rickettsial infection is the Weil-Felix assay. This is a conventional agglutination assay that has been used since 1916 (Cowan et al. 2009). It is based on the cross-reactivity of rickettsial species with antigens in strains of *Proteus spp* (Richards, 2012; Cowan et al. 2009). Its simple usage means that many developing countries still apply this test in their labs as the only diagnostic. However the assay lacks both sensitivity and specificity, resulting in many erroneous and unreliable results (Richards, 2012; Watt and Parola 2010; Wongchotigul et al. 2005; La Scola et al. 2000). Kularatne and Gawarammana (2009) compared the Weil-Felix test to the IFA, and the sensitivity was 33% whilst in an experiment by Wongchotigul et al. (2005) sensitivity in comparison to IFA was 47.3%.

iv. Enzyme-linked immunosorbent assay (ELISA). ELISA is the final serological method that can be used to diagnose rickettsioses and allows for the detection of specific antibodies, both IgM and IgG (La Scola et al. 2000). This method is more standardized than IFA, and allows for many patient serum samples to be tested at the same time. The diagnostic accuracy depends on how the positive threshold is established (Richards, 2012). ELISA is a genus specific semi-quantitative method that for rickettsioses should be used qualitatively only as it insufficiently monitors antibody titer changes (Chapman, 2006).

v. Another test proven to be useful in establishing an *R. conorii* diagnosis was a semiquantitative enzyme immunoassay, coined 'DS', which utilised dot-blot enzyme technology (Broadhurst et al. 1998). In this experiment soldiers sent to Botswana were tested using both IFA and the dot-blot enzyme immunoassay where sensitivity and specificity were shown to be 100% and 48% respectively. It concluded that, though not certain it was an *R. conorii* infection, it could have been *R. africae*, it was able to identify patient cases two days post-inoculation. IFA upon return to the United States confirmed *R. conorii* infection in all cases (Broadhurst et al. 1998). This rapid assay illustrates how in a situation where laboratory resources are scarce, a simple test can confirm infection. This allows for immediate treatment to take place, thus preventing further morbidity of the disease.

Diagnosis by molecular methods

Molecular diagnosis of rickettsioses is usually done by the polymerase chain reaction (PCR). Specific *Rickettsia* diagnosis by PCR can be performed in any lab that has the right equipment and to date has been widely implemented (Jensenius et al. 2004b). Primer sets are readily available, and target different rickettsial genes (Brouqui et al. 2004). Molecular methods are quicker and can be applied earlier than serology. PCRs can detect bacteria in blood, biopsies, and in vectors (Brouqui et al. 2007).

There is a variety of PCR that is available including nested PCR, qPCR and 'suicide' PCR. A nested PCR (nPCR) is a conventional PCR that is done twice in succession with a second primer pair to amplify a specific target in the product of the first

reaction. It is therefore more sensitive and specific than a single primer pair PCR (Althaus et al. 2010). Primers have been designed based on various specific rickettsial target genes. Notably the conventional (nested) PCR suffers from being prone to contamination as a major cause for false positive results. Quantitative real-time PCR, also known as qPCR, is a single tube assay that is less affected by contamination compared to the conventional approaches, while reaching a sensitivity that is similar or even better than that of the nested PCR (Richards, 2012). It gives the results in a quantitative way, allowing for comparison between samples and also allows results to be monitored in real-time (Angelakis et al. 2012). However, it requires a real-time thermocycler, which is not always available.

Fournier and Raoult first developed the suicide PCR in 2004 for use in rickettsial disease detection. This was done as an effort to improve the specificity that was reached by nPCR and qPCR. This technique is the same as the nested PCR but uses single-use primers that target single-use DNA fragments of the *Rickettsia* strains (Fournier & Raoult, 2004). The primer sequences are chosen from conserved regions of genes that are present in both *R. conorii* and *R. prowazekii*. To reduce contamination all reagents for the two consecutive PCRs are added to the reaction tube before the first amplification is done. Their results concluded a specificity of 100% and sensitivity of 68% for the suicide PCR, and also less contamination problems than seen in nPCR. They advise to use suicide PCR when rickettsiosis is suspected but cannot be confirmed with a conventional PCR (Fournier & Raoult, 2004). As with all other methods of diagnosing rickettsioses, administration of antibiotics prior to collection of samples significantly reduces the detection rate of molecular methods (Richards, 2012; Fournier & Raoult, 2004).

Rapid diagnostic tests (RDT)

There is little information published about RDTs and few are commercially available. In-house RDTs are commonly used in developing countries where more sophisticated serological or molecular methods are not possible. Those that are and where information has been published shall be presented here. The INDX Dipstick is a commercially available RDT, which uses *Rickettsia rickettsii* antigens to determine the cross-reactivity with other *Rickettsia* species. One study in Malaysia showed that the test was useful in diagnosing certain types of *Rickettsia,* for example rickettsial infection with 10 out of 12 *R. typhi* strains could be identified, but only 4 out of 10 *O. tsutsugamushi* cases were diagnosed (Koay & Cheong, 1993). Though screening of species in Malaysia proved to be achievable using the dipstick, a second serological test was recommended to confirm weaker reacting cases.

Blacksell et al. (2010) tested the accuracy of two separate RDTs in Laos; the first an immunochromatographic test to diagnose scrub typhus (ST ICT), the second an immunoblot test to diagnose murine typhus (MT IBT). They were tested mainly for purpose as point-of-care diagnostics of acute disease in rural settings. The specificity of both tests were high, however, general sensitivity of ST ICT and MT IBT tests were 34.7% and 48.4% respectively. This is relatively low and therefore the tests have limited usefulness for admission acute-phase specimens, which can be expected from the dynamics of the serological response. In later stages of the disease the sensitivity of both tests increased.

In 2011, Zhang et al. evaluated a new RDT for the detection of IgM and IgG antibodies against *O. tsutsugamushi*. IFA instruments are limited in China, and the diagnostic delay has caused for severe morbidity and mortality (Zhang et al. 2011). This is why the need for a reliable RDT is high in China. Case fatality rates up to 35% had been reported in the untreated local population. A combination of proteins from the different *O. tsutsugamushi* strains were used for antigens in the RDT and once patient sera was added to the test strip, results were available 15 minutes later. All sample sera was tested by both IFA and the RDT, and IgM and IgG sensitivities were tested separately and combined. 82 control samples and 33 scrub typhus patients' sera were used. Their results indicated that the RDT had a higher sensitivity than IFA namely 100% and 96.9% respectively. This concluded that the rapid test is suitable for early diagnosis in the acute phase of scrub typhus.

All examples of RDTs seem to be isolated experiments where results have not always proven definitive enough to cement the tests as commonly used diagnostic tools. Most results were however very promising. Due to the difficulty in detection methods, health care providers must usually rely on the clinical presentation such as the presence of an inoculation eschar, and include previous travel history of the patient and travel activity to make an initial diagnosis (Althaus 2010; Jensenius et al. 2004a).

Based on the abovementioned data I would advise to use molecular methods, namely nested PCR, for diagnosis in the early acute phase of a suspected *Rickettsia* or *Orientia* infection. This because it is not a lengthy procedure, can be undertaken in any lab in the Netherlands, and has a higher accuracy than serology. Furthermore I would advise this due to its higher sensitivity than serology in the early stages of infection. The preferred sample would be on an eschar or rash biopsy, as bacteremia is highest in these samples. Culturing would only be advised if it is absolutely necessary to identify the species or in the case of a severe infection. If a patient presents in the later acute phase, 7-10 days after fever starts or in the convalescent phase, I would recommend using IFA. If it were possible, I would combine it with Western blotting to conclusively identify the species and thus get a more accurate database of travel-associated rickettsioses. A convalescent sample should always be taken and tested as well in order to diagnose the infection with certainty. What should also be remembered is the infective species. If *R. africae* is suspected, an initial IFA could cause for false negative results due to delayed seroconversion.

Case studies

Only a few cases of rickettsial disease have been described in Dutch travellers returning from abroad. Most instances concern patients who have contracted *R. africae* after travel to regions in Africa, however two cases of murine typhus have also been noted. One case of each of these two diseases will be described here.

Tan et al. (2011) describe a 57-year-old Dutch male patient who presented after 4 days of malaise, nausea, joint pain and high fever. Symptoms had started after returning from a month long trip to Indonesia. Upon examination the patient presented with 39°C fever, blood pressure of 140/80 mmHg and a heart rate of 90 bpm. There was no presence of an eschar. A blood smear and quantitative analysis of buffy coat were done to exclude malaria and no discrepancies were noted. An initial diagnosis of typhoid fever was made and the patient was started on intravenous

ceftriaxon. However, in the following days no improvement was noted and a headache and rash appeared. After 9 days in hospital the patient deteriorated further by developing respiratory failure for which he was moved to the intensive care unit. Treatment with doxycycline and ciprofloxacine was commenced. Within a few days the patient's symptoms had improved and after two weeks in hospital he was released. Serological evidence later indicated infection with *Rickettsia typhi*.

Kager and Dondorp (2001) described a case of *Rickettsia africae* in a 26-year-old female patient who had noticed an itchy pimple next to her belly button 5 days after a trek through Kruger Park in South Africa. During this trek she had slept in the open air. Subsequently the pimple turned into a pustule, eventually developing into a red spot with a black crust. She had a painful swelling on her left groin and a few days later had high fever, a headache, and a stiff neck. She came to the hospital the following day where fever and eschar were confirmed and her neck muscles were tensed. Her entire body was further covered in 40 erythematous papules. Several lymph nodes in her groin were inflamed. The symptoms were considered characteristic of rickettsial infection and the patient was immediately put on doxycycline treatment. The next day her fever had disappeared and after two days the muscle pain and rash had mostly disappeared. Serology on the first sample taken 12 days after the patient's return from Kruger Park revealed no antibodies. In the second sample taken 14 days later antibodies against *R. africae* confirmed the diagnosis of African tick bite fever.

These cases illustrate that rickettsial infections can present themselves differently. In certain cases a distinct clinical picture is seen, whilst in another symptoms are non-specific. These two cases highlight the importance for health practitioners to be able to recognise the specific symptoms to allow for quick and accurate diagnosis.

Prevention

There are certain preventive measures that travellers can take to reduce the risk of infection. For example, repellents that contain *N,N*-Diethyl-meta-toluamide (DEET) can be applied to skin and clothing whilst other mosquito repellents have also shown some effect (Cowan et al. 2009; Jensenius et al. 2006). Furthermore, protective clothing should be worn such as boots (close-toed shoes), long trousers, and long-sleeved shirts are recommended. Whilst protective clothing certainly contributes to reduced infection risk, it should be noted that such recommendations are not very practical for warm weather conditions (Jensenius et al. 2003). Careful inspection of the body after trekking and hikes might be more practical but should be performed carefully, as ticks are small and difficult to identify. Less than 50% of patients infected with *Rickettsia* can remember seeing ticks on their body (Jensenius et al. 2003).

Recommendations and Conclusion

This review outlines the key diagnostic tools available to clinicians in the Netherlands for diagnosing *Rickettsial* infections. As mentioned previously, it is important for them to take swift actions when patients present with characteristic symptoms or a suspect travel history. My recommendation would be to always undertake at least one diagnostic test for rickettsioses if a patient present with febrile illness and a history of

travel to a rickettsiosis-endemic country. This because, though many cases are self-limiting, delaying a diagnosis or misdiagnosing could have severe effects on the patient.

If a patient is suspected of having a rickettsial infection I would recommend a clinician to perform either an IFA or nPCR as a preliminary diagnostic test. This along with any diagnostic tests for other suspected infections. Whether serology or PCR is done depends on the phase of infection the patient is in. I would advise IFA because patients presenting back in their home country will do so usually well after inoculation. This will normally occur when fever has been present for a few days, in which case IgM and IgG levels will be detectable for most rickettsial infections, with the exception of *R. africae.* However, PCR would be advised if patients are presenting in the early acute phase of the infection around days 1 to 7 after inoculation, or if IFA is negative and symptoms are characteristic. In such a case serology would be negative as IgM and IgG levels are not yet detectable. PCR also allows for identification of species, which can also be desirable in certain cases. In the case of both serology and molecular methods results can be seen the day of testing. If utmost certainty is needed in diagnosing a patient I would recommend using both serology and PCR, as together they provide strong evidence for or against infection. As previously mentioned, a convalescent sample should always be taken in patients to definitively confirm *Rickettsia* infection. The diagnostic test for a convalescent sample is always serological therefore IFA is once again recommended.

I would only suggest culturing if an infection is extremely severe and therefore knowing the species is of utmost importance. The culturing technique must always be done at a reference centre, is very laborious and results can only be seen after approximately 7 days. A few serological tests were discussed that are not advised for various reasons. ELISA is practical in large-scale studies where multiple samples are tested at the same time. It would therefore be ideal for epidemiological or surveillance studies, however for a single patient there is no advantage to IFA. The Weil-Felix test has been shown to lack both sensitivity and specificity in relation to other diagnostic tests. It would therefore not be recommended to use this if other tests are available.

For sampling I would advise to take an eschar swab or crust specimen as the ideal sample. This type of sampling is painless and quick, and has the highest concentration of bacteria ideal for testing. If there is no inoculation eschar present a rash biopsy would be the next best option. Bacteremia is still high in these samples and therefore it would also allow for the most accurate diagnosis. However, due to its invasive nature it is often not a desired sampling technique for patients. Blood samples are the final alternative and although it has a lower level of bacteria, can still act as a viable sample and can be used for all diagnostic techniques. It is also a sample that can always be taken regardless of the phase of infection the patient is in. A convalescent sample, which should always be taken around two weeks after initial sampling, will always be a blood sample as the eschar and rash will be gone at this point.

The importance of health care providers knowing about the different rickettsioses and recognising certain symptoms is also a goal of this review. It is essential that clinicians do not dismiss flu-like symptoms or rashes too easily in certain patients, when combined with travel history to *Rickettsia* endemic areas. It is thought that there is severe under diagnosis of rickettsial disease in international travellers. The

information currently available is based on over 450 published cases, however it is thought that many cases go unnoticed. This could be either due to pre-emptive treatment, serological tests done too soon and proving negative, or the self-limiting aspect of the disease. If this is combined with the knowledge that adventure tourism and ecotourism is also consistently increasing it can be predicted that the number of cases seen will be on a steady increase. It is therefore important that clinicians include *Rickettsia* infection as a differential diagnosis in the case of patients presenting with febrile illness.

There is a clear lack of sound and easily accessible techniques to be able to rapidly and successfully identify *Rickettsia* in patients. With expectations that the disease will be seen more often, good diagnostics is a necessity. Focus currently seems to be on expanding knowledge about the different species and their regions of endemicity. This is crucial in order to recognise the disease and diagnose it properly. This research should be continued and wider epidemiological studies should be done to be able to more successfully predict the scope of rickettsial infections. Certain travel-related epidemiological studies currently exist however they concentrate on certain rickettsioses or specific areas. For example, they focus on the tropics thereby excluding certain species such as Mediterranean spotted fever (Jensenius et al. 2009). Systematic and careful reporting of instances of infection should therefore be implemented. This would allow for an expansion of the "database" of cases, which could lead to increased information, and thus protection, of international travellers.

In conclusion there are many diagnostic tools available for clinicians to diagnose rickettsial infections. All of them have their clear advantages and disadvantages. Clinicians will always have to use their own judgement if a patient is suspected of infection due to the different manifestations of rickettsioses. They must always consider a broad range of infections in febrile patients, and *Rickettsia* infection should be part of the possible diagnoses. As treatment of rickettsial infections is mostly uniform I would always advise to use those tests that are available and give the most reliable results. With a suspected increase in the amount of *Rickettsia* infections a rapid diagnosis and treatment of these infections is a necessity.

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