3 Biotech (2020) 10:396
https://doi.org/10.1007/s13205-020-02389-w

REVIEW ARTICLE

Diagnosis of scrub typhus: recent advancements and challenges

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Received: 26 May 2020 / Accepted: 10 August 2020 / Published online: 18 August 2020

Abstract

Scrub typhus is a mite-borne, acute febrile illness caused by the bacterium *Orientia tsutsugamushi*. It is a re-emerging infectious disease of the tsutsugamushi triangle. Scrub typhus is transmitted through bites of contaminated chiggers (larval stage). Diagnosis of scrub typhus is challenging as its symptoms mimic with other acute febrile illnesses. Several methods are effectual for diagnosis of scrub typhus that includes enzyme-linked immunosorbent assay (ELISA), immunofluorescence assay (IFA), immunochromatographic test (ICT), Weil–Felix, polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP). Weil–Felix test was initially used for the diagnosis of scrub typhus in underdeveloped countries but not preferred due to a lack of both specificity and sensitivity. Other immuno-based methods like IFA and ELISA are most outrank for detection of scrub typhus due to their higher sensitivity and specificity, but not vigorous to lay bare the infection at early stages and need the convalescent sampling for verification of positive samples. On another deed, PCR based methods becoming acceptable over era due to its dexterity of early-stage diagnosis with higher specificity and sensitivity but lack its applicability in circumstances of scrub typhus due to the variegated genetic makeup of *Orientia tsutsugamushi* among its serotypes. The present review focused on various detection methods along with their advantages and disadvantages used in the diagnosis of scrub typhus. A comparison between available methods of diagnosis with challenges in the detection of scrub typhus is also summarized.

Keywords Biosensor · Immunochromatographic test · Immunoflourescence assay · Polymerase chain reaction · Scrub typhus · Tsutsugamushi triangle

Introduction

Scrub typhus is a zoonotic infection that ensues in an acute febrile illness inherent to the 'tsutsugamushi triangle' and caused by intracytosolic, gram-negative bacterium *Orientia tsutsugamushi* (Paris et al. 2013; Prakash 2017). Humans are the fortuitous host of *Leptotrombidium* spp. and dead-end host of *Orientia tsutsugamushi* and it is transmitted to the community through bites of *Leptotrombidium deliense* (Shivalli 2016). *Leptotrombidium deliense* is a species of chigger mite and the principal vector of scrub typhus disease. This ailment is commonly endemic to the 'tsutsugamushi triangle', but few studies recite its presence beyond the triangle including orientia-like species of bacteria in southern Chile and a lately recognized species *O. chuto*, in the middle east (Weitzel 2016). Scrub typhus is endemic in Southeast Asia and according to recent study annually 1 million cases found alone in this region (Saraswati et al. 2018). A higher prevalence of this disease is found in rural areas of Southeast Asia including India, Thailand, Korea, Australia,

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Russia, The Pacific Islands, and Japan. According to passive national surveillance systems, seroprevalence frequency of this infection is in between 9.3% and 27.9%, and mortality rate varied as high as 30% or more among untreated individuals (Saraswati et al. 2018; Bonell et al. 2017).

Symptoms of scrub typhus appear after 5–14 days of Leptotrombidium bite with manifestations of infection such as fever, rash, myalgia, lymphadenopathy, nausea, vomiting, eschar (black spot arises at mite biting site), abdominal pain, and non-specific flu-like symptoms. This infection precedes to severe complications that event in multiorgan failure including jaundice, acute renal failure, and disseminated intravascular coagulation (DIC), acute respiratory distress syndrome, myocarditis, and meningoencephalitis (Xu et al. 2017a, b). Symptoms of scrub typhus concur with other co-endemic diseases such as leptospirosis, dengue, brucellosis, and typhoid, which makes it more troublesome to differentiate from others (Koh et al. 2010). The presence of eschar at mite biting site is a specific (98.9%) marker for clinical diagnosis of scrub typhus; however, the presence of eschar can be varied extensively in patients from 7 to 97% (Saraswati et al. 2018). The presence of eschar in India and other Asian populations is meager, which makes it an inappropriate method for the detection of *Orientia tsutsugamushi,* hence, the diagnosis relies upon laboratory tests (Shivalli 2016). The laboratory-based diagnosis of scrub typhus relies on serological assays like the Weil–Felix test, indirect Immunofluorescence assays, indirect immunoperoxidase assays, enzyme-linked immunosorbent assay (ELISA) and immunochromatographic tests (ICT), etc. Among all serological assays, IgM ELISA-based method is most reliable for the diagnosis of scrub typhus (Phetsouvanh 2013).

On the other deed, a molecular-based approach like polymerase chain reaction (PCR) based diagnosis having more specificity and sensitivity for disease diagnosis. However genetic variations among strains of *Orientia tsutsugamushi* are the biggest defiance for its diagnosis using the genetic marker via PCR. More than 20 antigenically distinct strains have been recognized till now including Karp, Kato, and Gilliam. Antigenic variations among these strains have been associated with a highly diverse *56-kDa type specific antigen* (*tsa*) immunodominant gene (Kelly et al. 2009). It encodes a primary immunogen (type-specific antigen) located on the outer membrane surface of *Orientia tsutsugamushi* and accountable for eliciting neutralizing antibodies (Blacksell et al. 2008). Apart from this gene, few other genes have also been recognized as a genetic marker that comprehends *47-kDa high-temperature requirement A* (*HtrA*) and *16S rRNA* gene. *Htra* was initially considered as a conserved gene among the *O. tsutsugamushi* strains, but now it is evolving as one of the influential tools for the diagnosis of scrub typhus (Izzard et al. 2010; Jiang et al. 2013). So it is very essential to understand antigenic diversity between distinct strains of *O. tsutsugamushi* for the evolution of precise diagnostic tools. The present study focused on exploring various diagnostic methods used for the detection of scrub typhus, their advantages, and disadvantages.

Epidemiology of scrub typhus

Scrub typhus is highly endemic to "tsutsugamushi triangle" and covers more than 8 million square kilometers of area, expand from far eastern Russia in the north, to Pakistan in the west, Australia in the south, and Japan in the east (Xu et al. 2017a, b; Kuo et al. 2012). As per different studies, the majority of the scrub typhus cases have been reported from the tsutsugamushi triangle (Table 1). But neoteric studies comprehend that scrub typhus is no more restrain to the tsutsugamushi triangle and spreading across the globe (Kuo et al. 2012; Jiang and Richards 2018). Several factors like the presence of scrub vegetation, woodpiles, and the cattle around the residences have been considered as major factors for the acquisition of scrub typhus. A study reveals that as compared to behavioral and demographic factors, environmental factors seem to be the most prominent factor responsible for the occurrence of scrub typhus. Living in the vicinity of the water body, cooking outside the home, domesticating pets, and the presence of scrub vegetation in the vicinity of the house all increase the risk of scrub typhus (Lyu et al. 2013; Varghese et al. 2016; Rose et al. 2019). There are two mechanisms for the transmission of *O. tsutsugamushi*, i.e., transovarial (transmission of *Orientia tsutsugamushi* from the female to offspring through eggs) and transstadial (passage from mite larva-nymph-adult) (Phasomkusolsil 2009). Both methods fall in the category of vertical transmission and no shreds of evidence reported so far for horizontal transmission (a mite acquires Orientia from an infected host, and its offspring infect other hosts) of *O. tsutsugamushi* (Frances et al. 2000; Lerdthusnee 2002). In the Asia Pacific vicinity, primarily the cases were found in the southwest, southeast coastal and eastern regions of China. In China, cumulative incidences were found in the age group of 60–69-year-old, maximum in the month of June and July. Most of the cases were reported in the age group of 50–60-year-old individuals (23.36%) without any incidence differences in genders (Zhang et al. 2013a, b). In contrast to China, the highest outbreaks of scrub typhus in Japan were reported in the month of November (Ogawa 2002). The age distribution was also different from China, as maximum cases (62%) were reported in the age group of 51–75-years-old. Scrub typhus has been recognized as one of the most occurring rickettsial diseases in South Korea

Table 1 Prevalence of scrub typhus in different endemic areas of tsutsugamushi triangle with higher incidences age group, the effect on gender ratio and most common carrier mites present in these areas

S. no	Country	Region of prevalence	Peak season	Age group (higher incidences) (years)	Primary vector	Gender ratio	References
1.	China	Southwest, southeast coastal and eastern regions	June and July	50–60	*L. deliense*, *L. gaohuensi*, and *L. scutellare*	No differences	Zhang et al. (2013a, b), Mingyuan et al. (1987), Zhang et al. (2010)
2.	Japan	Kyushu, Tohoku-Hokuriku and Kanto	Nov and May	51–75	*L. scutellare*, *L. akamush*, and *L. pallidum*	No differences	Ogawa (2002)
3.	South Korea	Urban areas and northern regions	Oct and Nov	60–69	*L. scutellare* and *L. pallidum*	More female patients reported than male	Kweon et al. (2009), Kim et al. (2010), Park et al. (2015)
4.	India	South India, Northeast India, and Northwest India	Aug and Oct	–	*L. deliense*	No differences	Kelly et al. (2009), Varghese et al. (2006), Tattersall (1945), Isaac et al. (2004), Dass et al. (2011), Chrispal et al. (2010), Khan et al. (2012)
5.	Thailand	Northern Thailand	Tropical climate	50–59	*L. deliense*, *L scutellare*, *L.chiangraiensis* and *L.imphalum*	More male patients reported than female (2:1)	Kelly et al. (2009), Traub et al. (1954), Wongprompitak et al. (2013), Manosroi et al. (2006)
6.	Vietnam	North Vietnam	Summer	–	–	–	Nadjm et al. (2014), Le Viet et al. (2017)
7.	Australia	The tropical coastal periphery of northeastern Queensland and the adjacent Kimberly region of Western Australia	–	–	*L. deliense*	–	Currie et al. (1993), Faa et al. (2003), Unsworth et al. (2007), Odorico et al. (1998), Campbell and Domrow (1974)
8.	Taiwan	Central and eastern Taiwan especially in Nan-tou county, Hualien County and Taitung County and southwestern Taiwan	May–April and October–November	50–59 and 60–69	*L. deliense*	More female than male	Kuo et al. (2011)
9.	Philippines	Archipelago, primarily on Luzon	–	–	*L. deliense*	–	Reisen et al. (1973), Traub and Wisseman (1974)
10.	Malaysia	States of peninsular (west) Malaysia and in Sabah and Sarawak in east Malaysia	–	–	*L. deliense*, *L.arenicola* and *L.totrombidium*	–	Audy and Harrison (1951)

and accounts for 27.7–51% of total acute febrile illness patients and there is a unique observation of gender inequality, i.e. more female patients than male (Chang 1995). The gender equality ratio of Japan is in agreement with Taiwan where more female to male ratio has been reported with the higher cases in the age group of 50–60-year-old (Kuo et al. 2011). The peak months of disease occurrence in South Korea were in agreement with Japan, i.e., October and November with the highest frequency in the age group of 60–69-year-old patients, 27.48% (Kweon et al. 2009; Lee et al. 2015). In India, scrub typhus is still an under-diagnosed disease with disease occurrence all over the country from South to Northeast and Northwest India. The peak season of disease occurrence was reported between Augusts to November and mainly belongs to rural areas in India (Sinha et al. 2014; Varghese et al. 2006). Sero-epidemiological studies reveal a higher prevalence of scrub typhus in Thailand with a substantial increase in the cases from the 1980s to 2000s. The male to female gender ratio in Thailand was reported as 2:1 which is different from other countries like China and Japan with a higher occurrence of disease (22.3%) in the age group of 30–39-year-old patients (Suputtamongkol et al. 2009). In other countries of Southeast Asia like Vietnam, the disease was reported during the Vietnam War in the 1960s, and most of the patients diagnosed were army personnel. But the disease was neglected until the beginning of the twenty-first century, that creates gaps in publications and leads to insufficient data collection for epidemiological studies (Deller and Russell 1967; Thiebaut et al. 1997). However the recent studies in Vietnam reveal that the cumulative incidence of scrub typhus was found 1.1% among the general population, and ~ 3.5% among patients admitted with no significant difference between rural and urban areas (Vu Trung et al. 2017; Sinha et al. 2014). Few other countries of "tsutsugamushi triangle" also found endemic to scrub typhus that includes Indonesia, Malaysia, Philippines, Pakistan, Far East Russia, the continent of Australia, and recognized as "coastal fever" earlier in 1913 and later as scrub typhus (Xu et al. 2017a, b; Philip et al. 1946; Graves et al. 2006; Corwin et al. 1999; Nelyubov 2002; Urakami et al. 1999; Traub et al. 1967; Currie et al. 1993). Both serological (Thiga et al. 2015; Maina et al. 2016; Horton et al. 2016) and molecular studies (Cosson et al. 2015; Kolo et al. 2016) confirmed the presence of scrub typhus outside the traditional endemic areas, leads to the supposition that scrubs typhus is no more restricted to the traditional endemic area of "tsutsugamushi triangle" and becoming a global issue. Epidemiological studies play a major role in understanding the disease distribution in different regions, gender and age groups to control the disease. Spreading of scrub typhus outside the endemic areas intimate the possibilities of new *Orientia* species, and potentially new vectors for scrub typhus, that has yet to be identified.

Pathogenesis

Scrub typhus in humans is caused by the larval bite of trombiculid mite that harvests the bacteria (*Orientia tsutsugamushi)* in its salivary gland (Kadosaka and Kimura 2003). In this, infection and activation of endothelial cells assist in the morbid physiology of Scrub typhus and involved in infection of several organs including heart, kidney, skin, pancreas, and brain. Scrub typhus infection precedes to fatal multiple organ failure in several cases (7–15%) in the destitution of appropriate antibiotic treatment (Mathai et al. 2003; Jeong et al. 2007; Varghese et al. 2014; Peter et al. 2015; Rajapakse et al. 2017). Pathophysiology of scrub typhus is still not evident; however, few studies narrate the role of immune-mediated processes along with direct bacterial damage responsible for the local or disseminated pathological mechanism (Paris et al. 2015). *Orientia tsutsugamushi* aims a wide range of cells in host with cell types including dendritic cells and activated monocyte cells for completing its replicative cycles and acts as a vehicle for spreading orientia in the lymphatic system (Paris et al. 2012). Attachment to the extracellular matrix (ECM) and host cells is required for the internalization of intracellular bacteria using high-affinity receptors. This bacteria aim different cells by interaction of its TSA56 antigen (56 kDa types specific antigen) with one of the host autotransporter domain proteins like SCaC and SCaA with fibronectin, a glycoprotein of the ECM. Transporter proteins SCaC and ScaA augment adhesion of *Orientia tsutsugamushi* to non-phagocytic cells (Cho et al. 2010a, b; Ha et al. 2011; Lee et al. 2008).

Several polysaccharides expressed on the surface of cells including HSPGs (Heparan sulfate proteoglycans) and ECM assist in attachment and internalization of intracellular bacteria (Ihn et al. 2000). Some elements were recognized that assist in bacterial ingress process including transmembrane glycoproteins like α5β1 co-localized Orientia by activating integrin-activated signal transduction pathways (Fig. 1) (Kadosaka and Kimura, 2003). There are different defense mechanisms involved in the diminishing infection of an intracellular bacterium. According to different studies, it has been hypothesized that the pro and antiapoptotic effects within different stages of host cell infection or dissemination may vary to prevent the spreading of *O. tsutsugamushi* (Diaz et al. 2018).

However, the mechanism behind the modulation of apoptosis by *O. tsutsugamushi* is still undiscovered and needs further studies to unveil the underlying mechanism. Another defense mechanism to repulse infection of an intracellular organism like *O. tsutsugamushi* is autophagy; a catabolic

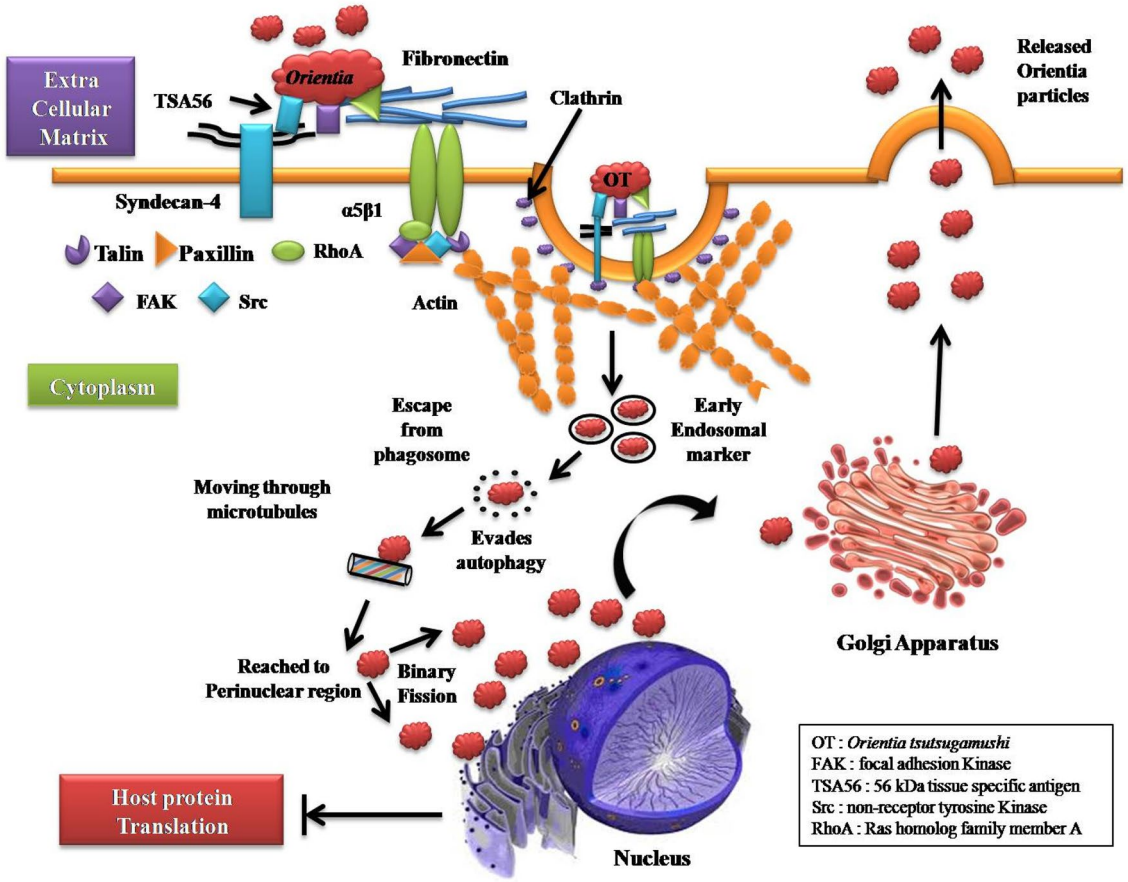

Fig. 1 Hypothetical model by reported events for the replicative cycle of *Orientia tsutsugamushi* in nonphagocytic cells

process that targets intracellular components for degradation by the fusion of endosomal and lysosomal compartments. However, *O. tsutsugamushi* can escape itself from the phagosomal compartment during endocytosis to destabilize the innate defense mechanism and to maintain its intracellular life cycle in the cell cytoplasm (Deretic 2012; Ko et al. 2013; Rikihisa 1984). Orientia imitates clathrin-mediated endocytosis for incorporation in human epithelial and fibroblast cells (Cho et al. 2010a). The entire mechanism behind the phagosomal escaping skill is still unknown, but few studies have revealed about the presence of hemolysin and a potential phospholipase D gene, whose expressed protein is similar to listeriolysin and phospholipase C proteins of Listeria monocytogenes involved in phagosomal escape process. So, studies also insinuate the possibility of these genes in *O. tsutsugamushi* replicative cycles in the cytoplasm (Cho et al. 2007, 2010b; Schnupf and Portnoy 2007; Stachowiak and Bielecki 2001; Ge and Rikihisa 2011). The Virulence factors involved in diminishing adaptive responses related to human scrub typhus infection are still not apparent, and the mechanism underlying it can be identified by further exploring the mechanisms of autophagy subversion.

Prevalent serotypes of *Orientia tsutsugamushi*

More than 30 serotypes of *Orientia tsutsugamushi* have been reported so far in endemic areas of tsutsugamushi triangle (Enatsu et al. 1999). Higher antigenic variation among serotypes was reported due to genetic diversity in 56-kDa type-specific antigen, a major outer- membrane protein (Tamura et al. 1995; Seong et al. 2001; Ohashi et al. 1992). *Karp*, *Kato*, *Gilliam*, *Boryong*, and *Kawasaki* are the most habitual serotypes of *Orientia tsutsugamushi* (Tamura et al. 1995; Yamamoto et al. 1986). The complete genome sequence of *Boryong* (NC 0094881) and *Ikeda* (NC 010793.1) serotype has been reported till now (Cho et al. 2007; Nakayama et al. 2008). Scrub typhus is highly endemic in the Asian-Pacific region (Table 2) extending from Afghanistan in the west to Japan in the north and from Russia in the North East to Australia in the south (Izzard et al. 2010). The Karp-like strain is highly predominant in the southeastern countries like Thailand (Blacksell et al. 2008; Wongprompitak et al. 2013; Ruang-areerate et al. 2011; Fournier et al. 2008), Vietnam (Duong et al. 2013; Le Viet et al. 2017), Taiwan (Lin

Table 2 Reported outbreaks of scrub typhus from different countries with prevalent serotypes and methods used for diagnosis

S. no	Year	Region	Out break	Method of diagnosis	Antibody/marker gene	Prevalent strain	References
India							
1	2018	Kerala	8 samples	ELISA	IgM	*Karp*-like *Kawasaki* like strain	Biswal et al. (2018)
				Nested PCR	*56-kDa tsa*		
2	2016	North-India	228 cases	ELISA	IgM	*Boryong*-like a prototype	Sharma et al. (2016)
3	2015	South India (Vellore), Northern India (Shimla) and Northeast India (Shillong)	263 eschar samples	ELISA	IgM	*Kato*-like strain (61.5%), *Karp*-like strain (27.7%) and *Gilliam* and *Ikeda* strain (2.3% each)	Varghese et al. (2015)
				PCR	*56-kDa tsa*		
4	2013	South India	154 cases	Weil–Felix test	OX-K	*Kato* (65%) *Karp* (30.7%)	Paris et al. (2015)
5	2011	Vellore	87 cases	ELISA	IgM	99% homology with strain *Taitung-2* and *TW461*	Prakash et al. (2011)
				ELISA	IgM		
6	2006	Himachal Pradesh	21 cases	Nested PCR	*56-kDa tsa*	Two different genotypes were identified with similarity in between *Karp* and *JP-1, Saitama* and *JG* type respectively	Mahajan et al. (2006)
				Weil–Felix test	OX19, OX2, OXK		
				MIF assay	IgG, IgM		
				Real-time PCR	*47-kDa HtrA*		
China							
7	2015	Shandong province	49 cases	Nested- PCR	*56-kDa tsa*	Most prevalent is the *Kawasaki* strain. *STA-07* is the second most common	Zheng et al. (2015)
8	2015	Northern China	43 cases	Nested- PCR	*56-kDa tsa*	*Kawasaki*	Zhang et al. (2015)
9	2013	Shandong province	3134 chiggers and 89 ticks	PCR	*56-kDa tsa*	*Kawasaki*	Zhang et al. (2013a, b)
10	2009	Northern China	36 cases	PCR	*56-kDa tsa*	*Kawasaki*	Liu et al. (2009)
Taiwan							
11	2011	–	130 mites	Nested PCR	*56-kDa tsa*	*Karp and Gilliam* like	Lin et al. (2011)
12	2010	–	116 cases	Nested PCR	*56-kDa tsa*	*Karp* like strain	Lu et al. (2010)
South Korea							
13	2012	–	44 eschar samples	PCR	*56-kDa tsa*	*Boryong* followed by *Kawasaki*	Jeong et al. (2012)
14	2011	–	69 cases	PCR	*56-kDa tsa*	*Boryong*	Lee et al. (2011)
15	2010	–	153 cases	PCR	*56-kDa tsa*	*Boryong*	Park et al. (2010)
Thailand							
16	2013	–	430 samples	PCR	*56-kDa tsa*	*Karp*	Unsworth et al. (2007)
17	2011	–		PCR	*56-kDa tsa*	*Karp*-like and *Ikeda*-like strain	Ruang-areerate et al. (2011)
18	2008	–	195 cases	PCR	*56-kDa tsa*	*Karp*	Fournier et al. (2008)
Vietnam							
19	2017	–	32 patients	IFA qPCR	IgG and IgM *56-kDa tsa*	*Karp* (70.4%), *Gilliam* (11.1%) *TA763* and *Kawasaki* (7.4%) and *TA716* (3.7%)	Le Viet et al. (2017)
20	2013	–	41 cases	PCR	*56-kDa tsa*	*Karp*	Blacksell et al. (2008)
Sri Lanka							
21	2017	Ragama and Balapitiya	28 cases	Real-time PCR	*47-kDa HtrA*	*JP-1(Karp* related) followed by *Kato*	Premaratna et al. (2017)
				Nested PCR	*56-kDa tsa*	Close homology with *Kuroki-Boryong L04956*	

The study concluded that in most of the countries the karp like strains are most prevalent and other strains are mostly region specific like *Kato* in India, *Kawasaki* in China and *Boryong* in South Korea

56 kDa tsa 56 kDa tissue specific antigen encoding gene, *47 kDa HtrA* 47 kDa high temperature requirement A, *Proteus antigens OX-19, OX2, OX-K* OX-19, OX_2—*Proteus vulgaris*, OX-K—*Proteus mirabilis*, *qPCR* quantitative polymerase chain reaction, *IFA* immunofluorescence assay, *ELISA* enzyme linked immunosorbent assay

et al. 2011; Lu et al. 2010; Yang et al. 2012), Sri Lanka (Premaratna et al. 2017) and India (Biswal et al. 2018; Varghese et al. 2013). However, in South Korea and China, the higher predominance of Boryong serotypes (Jeong et al. 2012; Lee et al. 2011; Park et al. 2010) and Kawasaki- like strain (Zheng et al. 2015; Zhang et al. 2013a, b, 2015) were found, respectively. However, now scrub typhus is not restrain to its indigenous areas and spreading outside tsutsugamushi triangle, as cases are reported from the Middle East, South America, U.A.E (Dubai), where Kato-like and chuto-like strains are prevailing (Weitzel 2016; Izzard et al. 2010; Le Viet et al. 2017; Taylor et al. 2015). The sequence alignment

of chuto isolates with different strains of *O. tsutsugamushi* reveals its higher divergence within the *16s rRNA*, *56 kDa tsa* and, *47 kDa HtrA* gene. The sequence analysis using 16 s rRNA gene shows 98.5% sequence similarity with *O. tsutsugamushi* strains Ikeda, Kato and Karp, whereas other genetic markers like 47 kDa gene analysis show its 82.3% sequence similarity with Gilliam strain. The partial *56 kDa tsa* gene sequence alignment study shows its 53.1% similarity with *O. tsutsugamushi* strain TA686. The maximum divergence within *O. tsutsugamushi* strains is reported 1% in most of the cases within the 16s rRNA gene sequences and maximum divergence (1.5%) is reported in *O. tsutsugamushi* strain Shimokshi. However, the percent divergence within 16s rRNA gene sequences in chuto isolate is more than 2% and so considered as a new species. The divergence level of *O. chuto* is also higher with other *O. tsutsugamushi* strains using other genes (*47 kDa HtrA*, *56 kDa tsa* gene) sequence analysis (Izzard et al. 2010). In India, most of the studies reported the high predominance of Karp-like strain in the north (Himachal Pradesh) and southern (Kerala, Vellore, Andhra Pradesh) part of the country followed by Kawasaki-like strain (Biswal et al. 2018; Mahajan et al. 2006; Varghese et al. 2013). Apart from these, the Kato-like serotype is also prevalent in the north-eastern (Shimla, Shillong, and Vellore) province of India (Varghese et al. 2015). Recent studies reveal that *Orientia tsutsugamushi* clades isolated from North-India (Punjab, Haryana, Himachal Pradesh, and Chandigarh) were closely clustered with Boryong prototype (Sharma et al. 2016). Variation in serotypes of different geographical provinces suggested the need for further study to get a better perception of its distribution. Although Karp, Kawasaki, and Boryong prototype are most prevalent, several new varieties were also identified in regional areas (Prakash et al. 2011; Mahajan et al. 2006; Zheng et al. 2015; Ruang-areerate et al. 2011). Future studies should converge upon genotypic studies and information about predominant serotypes of *O. tsutsugamushi* for better development of point of care diagnostic devices.

Diagnosis of scrub typhus

Symptoms parity of scrub typhus fever with other febrile illnesses such as dengue, typhoid fever, leptospirosis, and murine typhus, makes it laborious to diagnose. So, it will become very troublesome to assure this infection without laboratory tests or there should be the presence of pathognomonic eschars (Koh et al. 2010; Kundavaram et al. 2013). Presence of eschar in patients aids in the clinical diagnosis of scrub typhus with higher specificity (98.9%), but lack its applicability due to a wide variation in its distribution (7.0–97%) among patients (Paris et al. 2013; Saraswati et al. 2018). Therefore, the diagnosis of scrub typhus demands presumptive as well as definitive tests for accurate detection of disease. Laboratory-based methods are more reliable and precise in the diagnosis of scrub typhus and can be classified as direct and indirect methods (Fig. 2). A direct method of diagnosis involves isolation and culturing of bacteria in cell lines like L929 (normal fibroblast cell line of the mouse), HeLa (cancer cell line), BHK21 (baby hamster kidney fibroblast cell lines), Vero lineage (isolated from kidney epithelial cells extracted from an African green monkey) and their DNA-based diagnosis using different genetic markers by PCR. Indirect methods of diagnosis include serological assays such as Weil–Felix test, immunofluorescence assay (IFA), enzyme-linked immunosorbent assay (ELISA) and rapid diagnostic assays (Bora et al. 2018; Lijuan et al. 2011; Koraluru et al. 2015; Zhang et al. 2011).

Indirect methods of diagnosis

Indirect method of diagnosis relies on the detection of antibodies propagate as a result of humoral immunity against *Orientia tsutsugamushi*. This method includes serological assays that are based on the detection of IgG and IgM in patients' blood sample (Anitharaj et al. 2016; Ching et al. 2001; Blacksell et al. 2007). Immuno-based methods are mostly promote for detection of scrub typhus as direct method like culture and PCR-based assays required technical expertise and sophisticated labs and instrument facility (Ogawa et al. 2017). In Japan, it is recommended to use at least five serovars, viz. Kato, Karp, Gilliam, Kuroki (Yamamoto et al. 1989), and Kawasaki (Yamamoto et al. 1986) of *Orientia tsutsugamushi* for detection of scrub typhus. However, Karp, Karp-like, and Gilliam-like strains appear to predominate throughout the region of endemicity (Kelly et al. 2009).

Most of the commercially available serological kits (DEI-ABL75, In Bios, Abnova, etc.) for scrub typhus diagnosis are based on IgG- and IgM-based detection. In these assays, whole-cell proteins of Karp, Kato, and Gilliam, or their recombinant proteins such as 56-kDa tsa, 47-kDa HtrA, and 22-kDa scrub typhus antigen proteins, as well as cell-surface proteins (ScaA and ScaC) are used as an antigen (Kocher et al. 2017; Jiang et al. 2003). IgM based detection methods are the recommended one, due to their association with primary or acute infection, while IgG-based methods are not able to differentiate between a recent and past infection, but useful for determining the prevalence of scrub typhus, due to their long persistence in blood. So paired serum samples (acute and convalescent) are always recommended in serological assays for better support in the detection of scrub typhus (Sanap et al. 2017). The direct immuno-based methods include several techniques such as Weil–Felix test, indirect enzyme-linked immunosorbent assay (ELISA), immunofluorescence assay (IFA), immunochromatographic

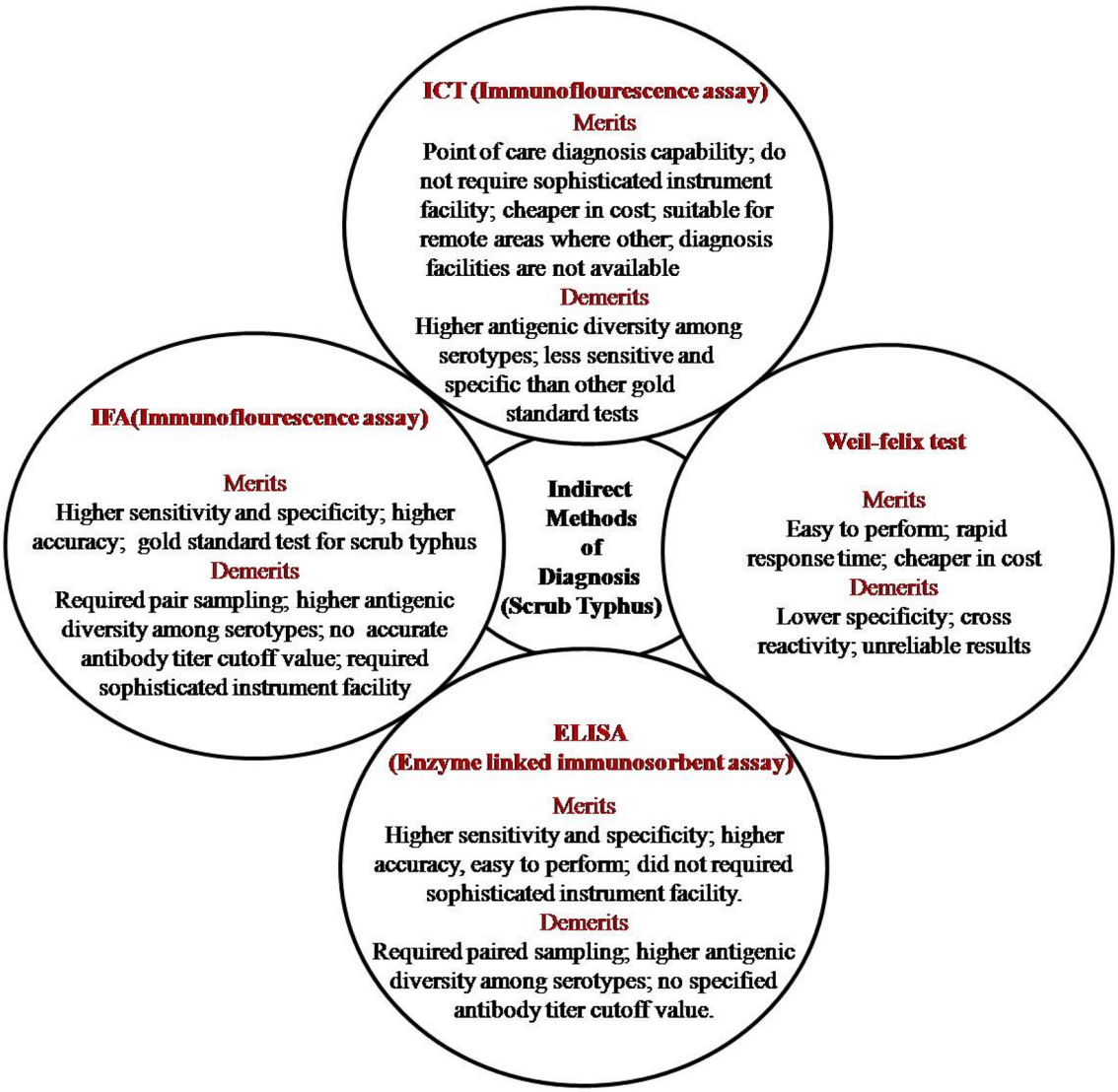

Fig. 2 Indirect methods for detection of scrub typhus with their advantages and drawbacks

test (ICT), and immunoperoxidase assays. All these methods have some advantages and disadvantages in the detection of *Orientia tsutsugamushi*, (Fig. 2) that are listed below:

Weil–Felix test

Weil–Felix test is a well-established agglutination method for the detection of rickettsial infection in the patient's serum samples. Weil–Felix test detects IgM antibody apparent 5–10 days successive onset of disease indication. This method is based on the detection of host immune response against different *Proteus* antigens such as OX19, OXK, and OX_2 that cross-respond with rickettsiae (Sanap et al. 2017). Certain strains of *Proteus* viz. *Proteus vulgaris* OX-19 and OX_2, *Proteus mirabilis* OX-K shared an alkali stable carbohydrate antigen with rickettsiae. So OX19 of *P. vulgaris* for typhus group rickettsiae and Rocky Mountain spotted fever, OXK of *P. mirabilis* for scrub typhus and OX2 for spotted fever infection are used for detection of rickettsial infection (Sanap et al. 2017). Weil–Felix test is a widely used rapid serodiagnosis method for the detection of rickettsial infections mostly in developing countries. Weil–Felix test has very less specificity and sensitivity due to the use of non-rickettsial antigen for agglutination. OXK reactive agglutinins appear 10–14 days after onset of scrub typhus and declined subsequently with time. Due to the pervasiveness of the *Proteus* species, lower serum titers are not considered significant until they are 1:160 or higher. However serum titer did not reach up to this level frequently, so only preliminary diagnosis can be made by a fourfold increase in titer during infection. Chances of false-positive results are also very high in the case of *Proteus* infection of the urinary tract,

leptospirosis, and relapsing fever. So negative Weil–Felix test results did not exclude scrub typhus and required a further diagnosis for confirmation. Despite of lower specificity, Weil–Felix is still widely used in small laboratories for initial screening of the scrub typhus in rural areas. In setups with inadequate diagnostic facilities, Weil–Felix test could be used as a primary screening method for qualitative estimation with clinical co-relation for better patients' management.

Immunofluorescence assay (IFA)

Immunofluorescence assay was initially introduced by Bozeman and Elisberg (1963) and later on modified to achieve similar performance with lower sample quantity using micro IFA technique (Gan et al. 1972; Robinson et al. 1976). The IFA Test uses fluorescein linked anti-human reporter antibody to detect the presence of scrub typhus-specific antibodies in the serum sample (Bozeman and Elisberg 1963). Karp, Kato and Gilliam's are the most frequently used antigens in indirect IFA for the diagnosis of scrub typhus (Blacksell et al. 2007). Indirect IFA considered as a gold standard test for the detection of scrub typhus due to its higher sensitivity and specificity (Ericsson et al. 2004). However other techniques like ELISA also have been reported with similar sensitivity and specificity with lower cost (Ericsson et al. 2004; Gupta et al. 2016). Like other serological tests, IFA-based detection requires the testament of raised antibody titer in paired samples. An antibody titer of more than 20-fold in a paired sampling (with a difference of 14 days in both sampling events) was considered positive (immunoperoxidase assay paper). Due to the retro prospective approach, paired sampling-based methods become unsuitable for early-stage diagnosis of scrub typhus. To overcome this limitation of paired sampling, region-specific standard antibody cutoff titer values need to be evaluated for prompt diagnosis of scrub typhus. Several studies reported cutoff values ranging from 1:10 to 1:3200, but no harmony has been found between them (Lim et al. 2015a, b). However, along with standard antibody titer cutoff value, the selection of antibody isotype and serovars (used as an antigen) specific to an endemic area also plays a major role in an accurate diagnosis. So it is suggested to use IFA for seroepidemiology study only in the endemic areas where seroprevalence has already been well established.

Indirect immunoperoxidase assay (IPA)

Immunoperoxidase assay is similar to IFA with the only substitution of fluorescein with a peroxidase enzyme that reduces its cost due to the elimination of expensive equipment such as a fluorescent microscope. Immunoperoxidase assay has several advantages over the IFA technique and widely applied in diagnostic virology (Kurstak et al. 1977). The indirect IPA method was evaluated for the diagnosis of scrub typhus using a smear of infected mice spleen with each strain of *O. tsutsugamushi* and found to be an alternative for IFA with the advantage of permanent preparations for re-examination and ability to observe infected and uninfected cells. IPA shares an advantage with IFA that any serotype can be used as an antigen and can measure either IgG or IgM individually. IPA could be used as an alternative test for IFA in areas where sophisticated instrument facilities are not available. However, its subjective readings make it inferior to other serological methods such as ELISA.

Enzyme-linked immunosorbent assay (IgG and IgM)

ELISA is most outrank among all serodiagnosis methods for the detection of scrub typhus due to their better sensitivity and specificity. Most of the ELISA based methods targets IgM and IgG antibodies in serum samples for the detection of *Orientia tsutsugamushi* (Rahi et al. 2016). IgM captured ELISA is a better method for the detection of scrub typhus as well as other rickettsial infections at the early stages of disease development (Rahi et al. 2016; Janardhanan et al. 2014). In *Orientia tsutsugamushi* infection, IgM antibodies titer can be observed after the 1st week of infection, while IgG antibodies appear at the end of the 2nd week. So IgM-based ELISA methods are better for early detection of scrub typhus and can differentiate nascent infection with the past one. Previous ELISA-based methods use whole purified cells lysate as an antigen for the detection of scrub typhus using serovars, Karp, Kato, and Gilliam or their recombinant proteins (Kocher et al. 2017; Jiang et al. 2003). Most of the advancements in ELISA-based diagnostic methods have been done by the use of recombinant antigens for the detection of scrub typhus with higher specificity and sensitivity (Blacksell et al. 2007; Ching et al. 2001; Coleman et al. 2002). Few studies suggest to use the combination of recombinant proteins from additional strains such as Karp, TA763 (r56C1), Kato (r56Kt), and Gilliam (r56Gm) to improve specificity and sensitivity of detection and to prevent false-positive results (Chao et al. 2011; Yang et al. 2019). The fact was assisted by a study that evaluated the sensitivity and specificity of the three recombinant proteins-based ELISA and resulted as a better approach in comparison to the present diagnosis methods. Several commercially available kits have been designed using the same approach such as InBios and evaluated in terms of sensitivity and specificity. The sensitivity and specificity for IgG and IgM were reported greater than 85% in comparison to the present gold standard method, IFA (Zhang et al. 2011; Blacksell et al. 2016; Silpasakorn et al. 2012; Chao et al. 2017; Kingston et al. 2015).

Another method was developed by modifying a Dot-ELISA using a combination of recombinant proteins to diagnose scrub typhus. Several studies evaluated the ELISA-based methods for their sensitivity and specificity and considered it as good as the gold standard method (IFA) available for the detection of scrub typhus (Rodkvamtook et al. 2015). Even though IgM-based immunofluorescence assay is a gold standard test for the diagnosis of scrub typhus, it has some drawbacks like its higher cost, the need for trained personal, and the requirement of a fluorescent microscope. So IgM-based ELISA may become a better alternate and possibly viable option for resource-limited endemic countries (Gautam et al. 2020).

A recent study focused on the identification of B cell and T cell epitopes on the surface protein HtrA (high-temperature requirement A) with higher conservation among *Orientia tsutsugamushi,* to use it as a target for vaccine development as well as for serodiagnosis. They have identified B cell epitopic peptide region that was highly conserved among most prevalent serovars of *Orientia tsutsugamushi* and suggested its further use in the development of a highly specific and sensitive diagnostic method for scrub typhus. Further studies are required to validate the finding to sort out the problem of antigenic diversity among *Orientia tsutsugamushi* strains and make its diagnosis easy and reliable by improving specificity and sensitivity (Sankar et al. 2019). The main drawback of the immuno-based methods is the lack of standardization and the choice of reference test to compare the ELISA, bring into question the validity of cut-offs used in diagnostic accuracy studies and observational studies. Further studies will be required to determine the region-specific cut-offs to improve the accuracy of the diagnosis method. To determine the area-specific cutoff, studies need to be done in different geographical locations, and considering the background antibody levels can differentiate the diseased from healthy individuals (Saraswati et al. 2019).

Immunochromatographic test (ICT)

ICT has been considered a point of care diagnosis system for the detection of scrub typhus with reported sensitivity and specificity similar to the other standard methods used for the detection of scrub typhus. ICT uses a recombinant mixture of 56-kDa outer-membrane proteins of *Karp, Kato* and, *Gilliam* strain as captured antigen for detection of IgG and IgM antibodies to *Orientia tsutsugamushi*. However, nowadays, the recombinant mixture from five different strains *Karp, Kato, Gilliam, Boryong,* and *Kangwon* with some regional strains is also being used as a captured antigen to detect scrub typhus for improving the specificity of the diagnosis methods in different endemic areas (Kim et al. 2013). An ICT-based assay for detection of scrub typhus has been reported with better sensitivity and specificity (96.8% and 93.3%, respectively) for the detection of IgM, whereas the poor specificity was reported (71.4%) for total antibody detection in the same study (Blacksell et al. 2010). A study used InBios rapid kit (ICT) for analyzing sensitivity and specificity of the ICT assay resulted in good sensitivity and specificity of 99.25%, 93.02% and 92%, 95% in India and Thailand, respectively (Kingston et al. 2015). Another study also shows the higher sensitivity and specificity for IgM (98.6% and 98.2%) than IgG (97.1% and 97.7%) using ICT assay (Immune Med RDT kit) with no cross-reactivity with other diseases (Diao et al. 2018). ICT has higher sensitivity and specificity than Weil–Felix and passive Haemagglutination assay and even significantly better specificity than IFA according to Bayesian latent class models (Sankar et al. 2019; Lim et al. 2015a, b). It has been demonstrated that the sensitivity of ICT increased by 20% when used in combination with DNA-based loop-mediated isothermal PCR assay (Blacksell et al. 2012). To achieve the higher sensitivity and accuracy in the diagnosis of scrub typhus, a surface-enhanced Raman scattering-based lateral flow assay (SERS-LFA) has been developed (Lee et al. 2019). The developed assay can perform the quantitative analysis of samples, which is not possible with the normal lateral flow assay. In this assay, the strips were made up of nitrocellulose membrane immobilized with recombinant 56 kDa antigen of *O. tsutsugamushi* as a capture probe on the test line. The SERS nanotags conjugated secondary Abs (Anti-human IgG) were used to monitor the Ag-Ab (target IgG in patients sample) interaction on the test line of the strip, which is further analyzed by the Raman spectrophotometer. The lateral flow assay strips (SERS-LFA) are designed to get integrated with a portable mini Raman spectrophotometer which makes it a better point of care diagnosis system. The developed lateral flow assay strips (SERS-LFA) were designed to get integrated with a portable mini Raman spectrophotometer which makes it a better point of care diagnosis system of diagnosis of scrub typhus. Therefore, ICT can also be used in combination with another assay to produce more accurate results. So it can be concluded that ICT assays have promising future in the diagnosis of scrub typhus due to its excellent sensitivity, specificity, field-deplorability, the rapid response time (within 10–15 min) without any requirement of sophisticated instrument facility. Further, it can be used as a diagnostic method especially in rural areas where the IFA facility is not available.

Direct methods of diagnosis

Direct methods of diagnosis involve bacterial isolation and culturing in chick embryos or established cell lines and

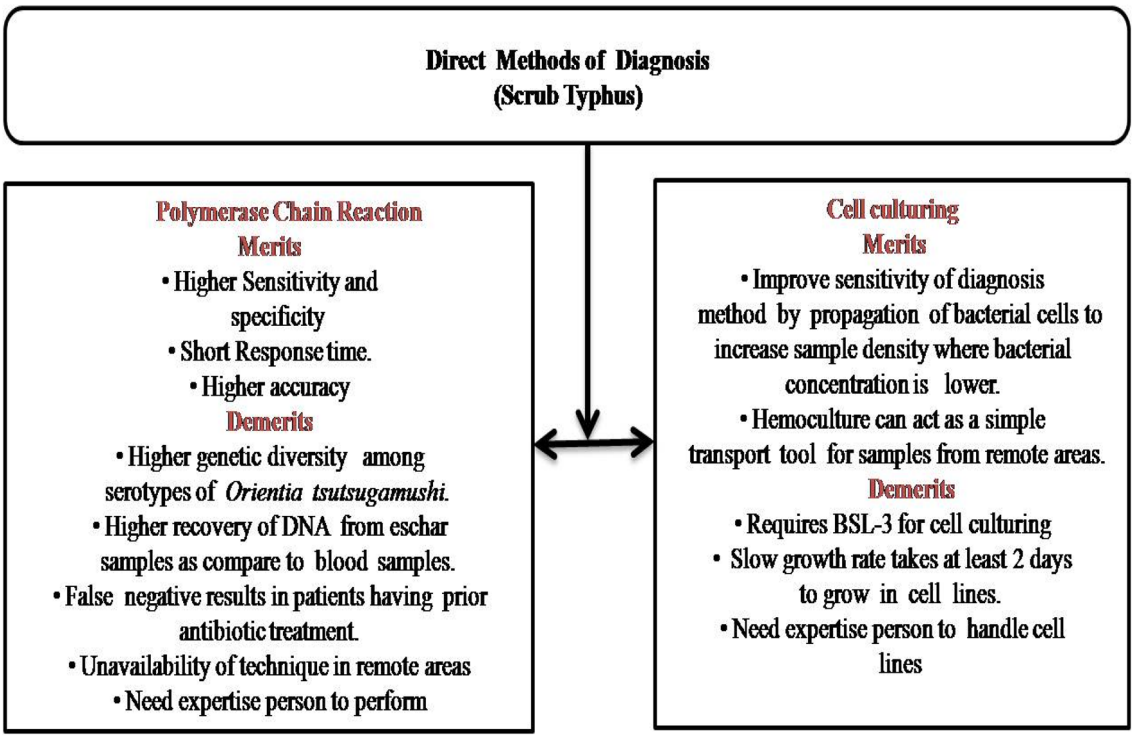

Fig. 3 Direct methods for detection of scrub typhus with their advantages and drawbacks

their detection using molecular assays such as PCR. In PCR several genetic markers such as *56 kDa tsa*, *47 kDa HtrA*, *GroEL*, and *16s rRNA* gene are targeted (Jiang and Richards 2018; Cho et al. 2000). Direct methods of diagnosis having advantages over indirect methods (Fig. 3) as it can detect infection in early stages with higher sensitivity and specificity. However, the direct method of diagnosis having several drawbacks like the requirement of biosafety level (BSL) 3 facilities for culturing of *Orientia tsutsugamushi,* higher genetic diversity among serotypes of scrub typhus (Koh et al. 2010; Paris et al. 2011; Paris and Dumler 2016; Kim et al. 2006; Luce-Fedrow et al. 2015). Advancements in direct detection methods along with their merits and demerits are demonstrated in the present study.

Bacterial isolation and culturing

O. tsutsugamushi targets endothelial cells, polymorphonuclear leukocytes, and macrophages for infection in patients (Seong et al. 2001; Cho et al. 2000; Choi et al. 2013). Invitro culturing (animal work or large culture volumes) of *Orientia tsutsugamushi* required high-containment biosafety level (BSL) three facility to propagate the bacterial cells using yolk sac of embryonated chicken eggs and established cell lines such as L929 (normal fibroblast cell line of the mouse), HeLa (cancer cell line), BHK21 (baby hamster kidney fibroblast cell lines), Vero lineage isolated from kidney epithelial cells of African green monkey (Tamura et al. 1995). Low risk activities including inoculation of cell cultures using low volumes/concentrations and invitro growth of culture at early stages can be performed in BSL-2 laboratories within a biosafety cabinet. Conventional haemoculture was considered as a better alternative in lower bacterial load (Dittrich et al. 2016). This growth capability of *Orientia tsutsugamushi* in a conventional hemoculture method can be utilized to improve the sensitivity of diagnostic techniques like PCR. A study suggested the possibilities of this conventional hemoculture technique based amplification for the development of an innovative approach to detect scrub typhus at the early stages of infection even after > 1 day. Further hemoculture can be used as a transportation tool for samples from remote areas to study *O. tsutsugamushi* diversity, which will aid in the development of more accurate diagnostic approaches (Dittrich et al. 2016; Varghese et al. 2015).

Polymerase chain reaction

PCR-based detection methods are highly specific and sensitive for disease diagnosis and can detect bacterial infection even in early stages, unlike serological methods. PCR based detection methods utilize genetic markers such as *56 kDa tsa*, *GroEL*, *16s RNA* and *47 kDa HtrA* to detect specifically the target organism, i.e., *Orientia tsutsugamushi* (Bora

et al. 2018; Jiang et al. 2004; Sonthayanon et al. 2009; Paris et al. 2009). The *56 kDa tsa* gene is mostly used for the characterization of the *O. tsutsugamushi* strains. Conventional PCR has the problem of sensitivity and specificity due to Human blood DNA contamination in isolated patients DNA sample as the bacteria has intracellular habitat. This limitation was overcome by identifying a conserved region of the *GroEL* gene and found more sensitive (68.2%) than other conventional PCR methods (Kim et al. 2011). Eschar samples-based PCR has better sensitivity and specificity in comparison to isolates from whole blood, buffy coat, and blood clots due to lower contamination of DNA sample (Saraswati et al. 2018). On another hand nested PCR based method has been reported with better specificity and sensitivity in comparison to conventional PCR (Kim et al. 2011).

In contrast to the above mentioned molecular approaches, real-time PCR-based methods are getting more attention now a day, due to their higher sensitivity, specificity and continuous monitoring capability of DNA amplification (Table 3). Real-time PCR-based detection methods of scrub typhus have already reported with targeted genes such as 47-kDa HtrA outer membrane protein gene (Saravanan et al. 2020) or 16S rRNA gene (using hydrolysis probes) or the 60-kDa heat shock *GroEL* gene (using SYBR green) (Jiang et al. 2004; Sonthayanon et al. 2009; Paris et al. 2009). However, a noble multiplex real-time PCR approach using several probes labeled with different fluorochromes was developed for the diagnosis of scrub typhus. This method was designed by targeting multiple genes such as genes encoding the 47-kDa antigen and the GroEL protein with human interferon beta (IFN-β) as an internal control and was found more sensitive and specific (sensitivity 86%, specificity 100%) than previously reported molecular assays. Multiplex approach either in conventional and real-time PCR is

Table 3 Comparative analysis of sensitivity and specificity of different techniques used for the diagnosis of scrub typhus

S. no	Diagnosis method	Biomarker (gene/antibody)	Specificity (%)	Sensitivity (%)	References
1.	ELISA	IgM	95.5	88.0	Saraswati et al. (2019)
2.	SERS-LFA	56 kDa tissue specific antigen	100	100	Lee et al. (2019)
3.	Real-time PCR	*HtrA*	70.5	69.7	Rawat et al. (2018)
	ELISA	IgM IgG	82.7 75	96.4 91	
4.	Nested-PCR	*groEL* *56-kDa tsa* *16 s rRNA*	– – –	68.2 27.6 20.6	Patricia et al. (2017)
5.	Multiplex PCR	*47 kDa htrA* and *groEL* gene	100	86	Tantibhedhyangkul et al. (2017)
6.	ELISA IFA	IgM IgM	94.12 93.5	100 100	Gupta et al. (2016)
7.	ELISA	IgM	91	93	Blacksell et al. (2016)
8.	Nested PCR Real-time PCR	*56 kDa tsa* *47 kDa htrA* *groEL*	98.4 96.1 93.0	56.8 63.2 71.4	Lim et al. (2015a, b)
	IFA	IgM	83.8	70	
	PanBio IgM ICT	IgM	96.8	72.8	
9.	ELISA Weil Felix	IgM IgM	95.5 93.3	85.3 67.1	Koraluru et al. (2015)
10.	LAMP Combination of Panbio IgM ICT and LAMP	– –	94 91	52 67	Blacksell et al. (2012)
11.	LAMP Real-time PCR Real-time PCR Nested-PCR	– *47 kDa htrA* *GroEL* *56-kDa tsa*	95 90 94 88	53 79 77 83	Paris et al. (2011)
12.	Nested-PCR ELISA	*56-kDa tsa* IgM	100 73	58 100	Prakash et al. (2011)
13.	Nested-PCR Q PCR Conventional PCR	*47 kDa htrA* *47 kDa htrA* *47 kDa htrA*	100 100 100	85.4 82.9 7.3	Kim et al. (2011)
14.	Nested-PCR (buffy coats)	*56-kDa tsa*	100	82.0	Kim et al. (2006)

SERS-LFA surface-enhanced Raman scattering-based lateral flow assay, *ICT* immuno-chromatographic test, *LAMP* loop-mediated isothermal amplification, *Q-PCR* quantitative PCR

becoming a better option for the detection of scrub typhus due to their increased specificity, as the genome of *O. tsutsugamushi* shows a high degree of genetic polymorphism. However real-time PCR method with the multiplex approach is more advantageous as it is more sensitive and specific with ability to monitor DNA amplification with a detection limit as low as ten copies per reaction, that makes it a remarkable approach for detection of scrub typhus even using blood samples (Tantibhedhyangkul et al. 2017). Real-time PCR-based method sorts out the problem of response time and specificity, but lacks its applicability due to its expensiveness and required sophisticated instrument facility. Regardless of the expensiveness, multiplex real-time PCR approach can become a standard test for the detection of scrub typhus (Dittrich et al. 2016) and helps to study the prevalence of serovars in different endemic areas to find out the representative strains from different regions.

Another variant of PCR, i.e., LAMP has been developed for the diagnosis of acute scrub typhus infection, targeting the *groEL* gene, encoding the 60 kDa heat shock protein of *Orientia tsutsugamushi* (Kim et al. 2006). LAMP technique is based on isothermal amplification of DNA using polymerase and set of primer pairs that produce a hairpin DNA template. LAMP is highly specific and sensitive with better efficiency and rapid response time. It can detect the DNA concentration of 1 mg/ml within 60–90 min. The amplified product is determined via photometry for turbidity caused by the precipitation of magnesium pyrophosphate as a by-product of the reaction. The reaction can also be quantified in real-time by measuring the turbidity or by fluorescence using intercalating dyes such as SYTO 9. The validity of the diagnostic assays can be evaluated on parameters of scrub typhus infection criteria (STIC) that includes (1) positive cell culture isolation of *Orientia tsutsugamushi,* (2) an admission IgM titer > 1:12,800, (3) a fourfold rising IgM titers in paired serum samples, (4) a positive result in at least two out of the three PCR assays using *56 kDa tsa*, *47 kDa HtrA* and *groEL*-based target gene (Fig. 4). LAMP method was compared with other methods mentioned in STIC for evaluation of its sensitivity and specificity of detection. The diagnostic accuracy of the LAMP assay was found similar to real-time and nested conventional PCR assays, but superior to the antibody-based rapid test in the early disease course (Paris et al. 2011). The combination of DNA- and antibody-based detection methods increased sensitivity with minimal reduction of specificity and expanded the timeframe of adequate diagnostic coverage throughout the acute phase of scrub typhus.

Future of scrub typhus diagnosis

Biosensor based disease diagnosis with higher sensitivity and specificity becoming the future of disease diagnosis. It is becoming a strong point of care diagnosis system for hasty and precise diagnosis of several diseases that are not facile to diagnose by other laboratory diagnosis methods (Kaushal et al. 2017; Gupta et al. 2017; Singh et al. 2017a, b). Biosensors composed of biospecific ligands, antibodies, nucleic acid receptors (Fig. 5) are categorized as affinity biosensors. Immunosensor is also a type of affinity sensor based on specific antigen–antibody interactions that

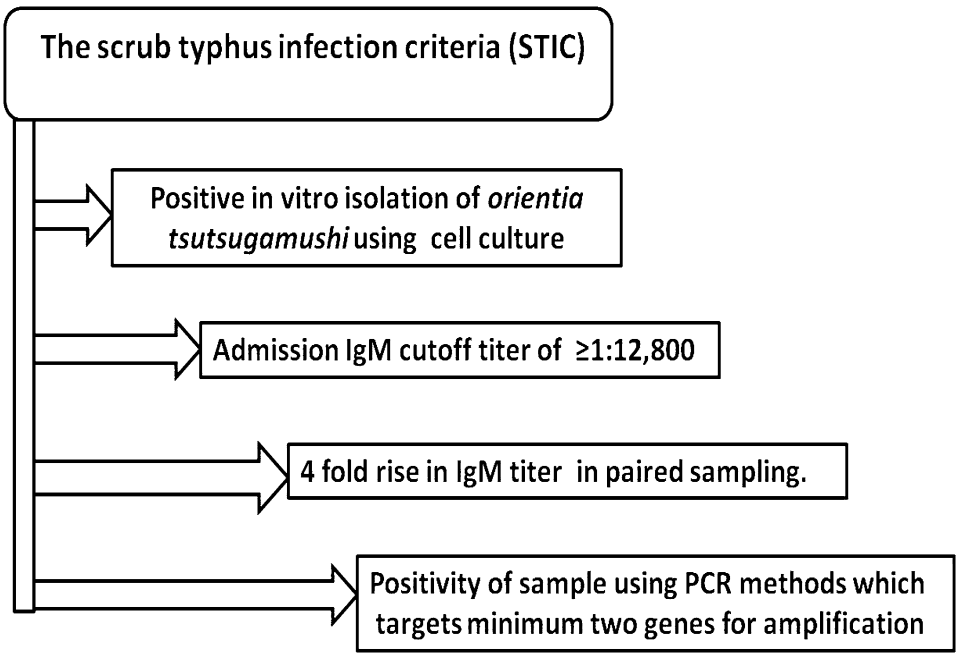

Fig. 4 The scrub typhus infection criteria (STIC) defined for accurate and reliable detection of *O. tsutsugamushi* (Paris et al. 2011). The STIC has been used to define the parameters on which a scrub typhus infection can be confirmed more specifically

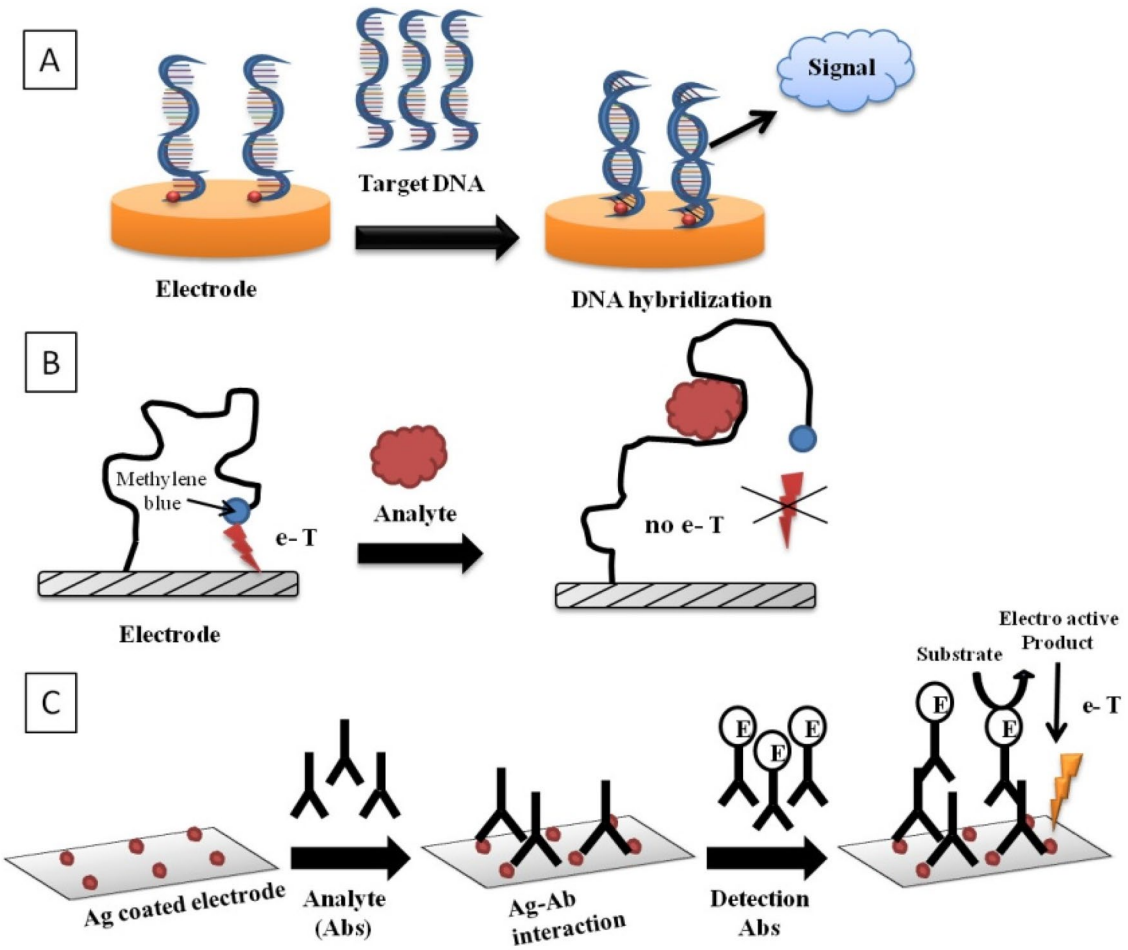

Fig. 5 Schematic representation of working principal of electrochemical affinity biosensors based on **a** nucleic acid probe, **b** Aptamers and **c** immuno-based approaches. All approaches are based on an analyte-receptor (Ag-Ab, DNA-DNA, Aptamer-complementary element) binding event which brings physiochemical changes on transducer (working electrode) surfaces like change in Ip (current) value in electrochemical sensor which is monitored by the potentiostat/galvanostat. The signal intensity is proportional to the analyte concentration in the sample

enable the detection of an analyte in a sample using label and label-free approaches. label-free approaches are better as it did not require the reporter molecule for monitoring Ag-Ab interaction and reduce response time by eliminating the extra incubation steps (Trindade et al. 2019; Gupta et al. 2017). However, the higher expenses of antibodies and their lower stability is its potential drawback. DNA sensor, another type of affinity biosensor based on the principle of DNA hybridization. It measures the physiochemical changes associated with DNA hybridization events to detect a wide range of analytes in samples including bacteria and viruses (Saylan et al. 2019). In recent years, DNA biosensor have gain more attention due to their higher sensitivity, specificity and shorter turnaround time (Franch et al. 2019). DNA biosensor is based on probe-cDNA interaction for the detection of specific DNA markers confined to a particular analyte (bacteria, virus) in the sample. The DNA hybridization induces physiochemical changes onto the transducer surface, that is measured by electrochemical, optical (surface plasmon resonance, fluorescence resonance energy transfer) and mass-based (surface acoustic wave, quartz crystal microbalance) transducers (Diao et al. 2018; Fu et al. 2019; Omar et al. 2018; Takalkar et al. 2017; Ozalp et al. 2015; Yao et al. 2017).

Among all transducers, electrochemical one is better due to their excellent sensitivity, selectivity, stability and lower limit of detection (Fu et al. 2019). DNA-based biosensor have some limitations as DNA molecules are sensitive towards several physical conditions like pH and temperature. Aptamers are a better alternative to normal DNA probe due to their high thermal stability and binding affinity (Song et al. 2012; Dalirirad and Steckl 2019). Aptamers are oligonucleotide sequences, which can bind with any target molecule with higher affinity through

electrostatic interactions, hydrophobic interactions, or their complementary shapes (Mallikaratchy 2017). Aptamers with an affinity for the desired target are selected from a large oligonucleotide library through a process known as the sequential evolution of ligands by exponential enrichment (SELEX). Aptamers can be developed against a wide range of targets from micro to macromolecules, giving it a wide range of applications (Han et al. 2010). Several advancements have been done so far in DNA based sensors to improve its performance like combining DNA sensor with other techniques like loop-mediated isothermal amplification for detecting meat adulteration onsite (Xu et al. 2017a, b), nanopore electrochemical biosensor based on hybridization chain reaction strategy for early diagnosis of cancer (Zhao et al. 2017) and amperometric biosensor utilizing a two-leg DNA walker for amplified electrochemical detection of nucleic acids, etc. (Wang et al. 2018). But further advancements are required in DNA-based biosensor that makes it a future point of care system for early-stage diagnosis of infectious diseases.

Scrub typhus is one of the under diagnosed and neglected diseases due to several reasons, among which genetic diversity of serotypes is a major issue. Biosensor can become an ultimate choice for the detection of scrub typhus due to their higher specificity and sensitivity. In DNA-based diagnosis approach, a highly conserved marker has to be identified for detection of scrub typhus as most of the DNA based methods are facing the problem of genetic diversity and lead to false-negative results. So targeting multiple DNA markers can solve this problem, as there will be a higher possibility of target site detection due to the presence of multiple binding sites. As an alternate combining LAMP with DNA biosensor can become a better approach for sorting out the problem of specificity and sensitivity of DNA-based methods. LAMP will target and amplify the template DNA more specifically and biosensor will monitor the hybridization and amplification event simultaneously with higher sensitivity and in less time.

On the other hand, the Immunosensor-based approach can become an alternative to the DNA sensor as recombinant antigen-based detection shows better sensitivity and specificity of detection. In the case of scrub typhus, immuno-based approaches having advantages over DNA based diagnosis approaches. New studies suggested using these immuno-based approaches to develop the integrated sensors to improve the sensitivity and specificity of the sensor. An effort has been made to develop a lateral flow assay (LFA) based sensor, which integrates the LFA strips with the miniaturized Raman spectrophotometer to make it a quantitative method feasible for field testing (Lee et al. 2019). So such integrated biosensors can be developed to overcome the limitations of present immuno-based approaches, which is facing the problem of poor stability and higher cost. Further studies are required to find out the representative strains of the different endemic areas, and the region-specific cut-offs for validation of immuno-based methods, which will aid in the diagnosis of scrub typhus by both approaches with more reliability.

Conclusion

In conclusion, DNA-based approaches are the future of early-stage diagnosis of scrub typhus, that needs to be improved by further studies. Compared to present diagnosis methods like Weil–Felix, ICT, ELISA, and IFA, the molecular assays are very rapid, specific, and sensitive and can be used as a rapid point of care system. The present review summarizes the advancement in the detection of *Orientia tsutsugamushi* in different biological samples so far. Most of the reports were focused on different techniques used for the detection of scrub typhus with their advantages and drawbacks. The review highlights the comparison between available methods of diagnosis with challenges in the detection of scrub typhus. The review also highlights the future possibilities for sorting out the issues of scrub typhus diagnosis that need to be evaluated using wet-lab studies for further validations.

Acknowledgements Author acknowledges Department of Science and Technology, (DST), Govt. of India for their grant (SP/YO/079/2017).

Author contributions DK and AK contributes a major part in the manuscript. All the other authors have contributed equally to the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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