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REVIEW ARTICLE

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Molecular remedies against *Cryptocaryon irritans* Brown, 1951—Practical difficulties

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Abstract

This review paper deals with the immunological response in marine fish infected by *Cryptocaryon irritans* and discussed the practical difficulties in the development of potential molecular remedies. Browsing through the literature, it is understood that the development of molecular diagnostic tool to detect the early infection in fish by *C. irritans* is under progress. Once it is successfully established, the possible application of this technique could be suggested for even the non-specific treatments such as copper-based medications and bare-bottomed quarantine of culture tanks. However, prior protection of healthy fish from *C. irritans* could be accomplished through proper immunization by infective theronts. In this case, a well-suited in vitro culture technique is necessary to harvest theronts in large scale. Recent immunological and transcriptome studies predicted that the components related to local immune response are more effective against *C. irritans* than their systemic counterparts. However, in vivo experiments to prove the effect of these antiparasitic molecular components are meagre. Though molecular vaccines, developed from the well-characterized 34 kDa immobilization antigen (iAg), responded well, these remedies fail to have a uniform impact against antigenically different strains (serotypes). More research is recommended to identify common protective epitopes from different serotypes. Also, studying the adjuvant effects of various innate responsive components like cytokines is significant. The suggested studies would be immensely helpful to develop effective vaccines against *C. irritans* infection and thereby optimize the aquaculture practices.

KEYWORDS

Cryptocaryon irritans, cryptocaryoniasis, immobilization antigen, immune response, molecular remedies

1 | INTRODUCTION

Cryptocaryoniasis caused by a parasitic protozoan *Cryptocaryon irritans* appears to be an emerging health problem in marine teleost fish of tropical and temperate waters causing their mass mortality and financial loss in both ornamental and food fish industries (Wright & Colorni, 2002). This histophagous parasite infects the skin, gill and fins of host fish (Nigrelli, 1966). Marine ecologists refer this obligate parasite as 'ich' or 'white spot', and the theront stage of this parasite will not survive for more than a day or two without the host fish (Iwama, Pickering, Sumpter, & Schreck, 1997; Dickerson & Dawe, 1995). Sikama (1937) reported first of its kind on *C. irritans* in a Bulletin of the Japanese Society of Fisheries Oceanography published from the Tokyo Imperial University Institute for Fisheries. In this report, the parasite infecting the marine fish was not scientifically identified and was only with a generic title 'a ciliate parasite' as

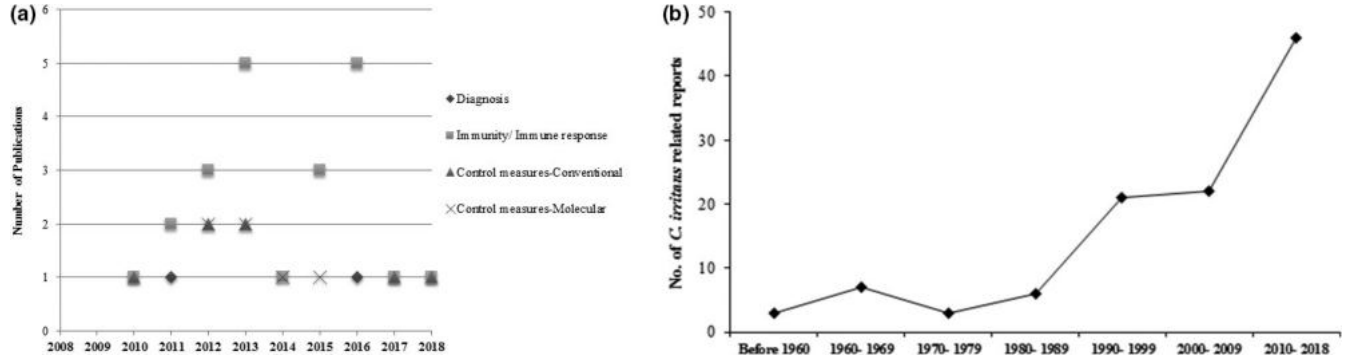

FIGURE 1 Chronological distribution of *Cryptocaryon irritans*-related research work conducted. a. Number of research papers deal with aspects such as diagnosis, immunity/immune response and control measures; b. decade-wise reports among the total number of research papers published

it showed extreme similarity with *Ichthyophthirius multifiliis* Fouquet, 1876 (Aphia ID: 730, 05, WoRMS taxon details), a ciliate protozoan parasitizing the freshwater fish. In his subsequent study entitled 'About Weisspünktchenkrankheit in marine fish' (Sikama, 1938), he named the disease caused by this parasite as 'white spot disease'. A decade later, Brown (1951) taxonomically described the parasite and named as *Cryptocaryon irritans* after it was sampled from the marine Aquarium of the Zoological Society of London.

In the period between 1960 and 1972, serious debate was going on regarding the naming of this parasite. Dashu and Lien-Siang (1960) called Sikama's ciliate as *Ichthyophthirius marinus* sp. nov. pro *Ichthyophthirius* sp. Sikama and the name was retained in the following publications of Sikama (Sikama, 1960, 1961, 1962). However, in the subsequent research papers from the worldwide, the parasite was named as *Cryptocaryon irritans* Brown, 1951 (Brown, 1963; Nigrelli, 1966; Wilkie, 1969). Canella (1972) opined the inappropriate naming of the species as *Cryptocaryon irritans* Brown, 1951. Instead of the term 'white spot disease', Wilkie (1969) coined the term 'cryptocaryoniasis' while publishing the outbreak of this disease in marine aquaria at Scripps Institute of Oceanography, San Diego, California.

In the review made first by Blasiola (1976), it was noticed that the studies on *C. irritans* from 1937 to 1969 exclusively focused on its occurrence. Cheung (1979) made first laboratory experiments on its encystment and excystment under different temperature and salinity conditions. Based on the information from this study, an attempt was made for the manual control of *C. irritans* in aquatic farms using hypersaline and chemical treatments (Huff & Burns, 1981). Basic information on the structure, biology, life cycle and control measures was also appeared in the subsequent reports (Cheung, 1981; Colorni, 1985, 1987; Xu, Jiang, & Cheng, 1992). Angelo Colorni and Mattthews made remarkable findings on the structural peculiarities of parasitic (trophont), reproductive (pro-tomont and tomont) and free-living (theront) phases of *C. irritans* through the transmission and scanning electron microscopic studies (Colorni, 1992; Colorni, & Diamant, 1993; Diggles, 1997; Maselli, 1994; Mattthews, Matthews, & Burgess, 1993). Colorni and Burgess (1997) also reviewed the progress in *C. irritans* research. More information on the ultra-structure of the parasite came out in the subsequent reports (Huang, Ma, & Li, 2005; Li, Huang, Ma, & Xie, 2006; Ma, Fan, Yin, Ni, & Gu, 2017; Ma, Li, Xie, & Huang, 2006; Watanable et al., 2016). Relevant reports pertaining to the occurrence, life cycle and host–parasite relationship of *C. irritans* and influence of environmental parameters on its development are also available (Burgees & Matthews, 1995; Colorni, 1985, 1987, 1992; Colorni, & Diamant 1993; Diggles et al., 1996; Diggles & Lester, 1996a, 1996c; How at al., 2015; Ishitani et al., 1996; Luo, Xie, Zhu, & Li, 2008; Mattthews et al., 1993; Ma et al., 2016; Sun et al., 2011; Yin et al., 2014; Yoshinaga, 2001).

Though *C. irritans* was previously included in the Class Oligohymenophora and family Ichthyophthiriidae due to its close morphological resemblance to *Ichthyophthirius multifiliis*, subsequent cytological studies failed to prove this. Molecular studies based on the phylogenetic analysis of 18S rDNA gene, *C. irritans*, was grouped under the Class Colpodea (Dickerson & Dawe, 1995; Diggles & Adlard, 1995; Wright & Colorni, 2002). Among the *C. irritans* isolates, high degree of intraspecific variation was found to be existed as it showed difference in morphological size, reproductive characters and incubation period for ex-cysting under different host and/or temperature conditions (Diggles & Adlard, 1997; Diggles & Lester, 1996b; Jee, Kim, Park, & Kim, 2000). Apart from this, strain differences were confirmed by sequencing the ribosomal DNA (rDNA) regions containing full/part of 18 S, the entire first internal transcribed spacer (ITS-1), full/part of 5.8 S of rDNA and full/part of second internal transcribed spacer (ITS-2) (Sun et al., 2006; Yambot, Song, & Sung, 2003). According to Chi et al. (2017), Cox-1 divergence could discriminate the *C. irritans* isolates into two main groups, which may also consist of many subspecies and/or syngens. Dickerson and Dawe (2006), in their review, focused on pathobiology of *Cryptocaryon irritans* including geographical distribution, host range and genetic susceptibility.

For the present paper, 109 research articles were reviewed and out of which 13 reports were on the taxonomic cataloging, 11 on the conventional control measures, 8 the molecular control measures, 5 presented methodologies for laboratory maintenance of the parasite and rest of the 72 reports focused on immune responses

against *C. irritans* (Figure 1). It also reveals that during the last two decades, 64.5% of reports on *C. irritans* were from China, 14.5% from Japan, 11.3% from Taiwan and less than 3% from the countries like Malaysia, USA, Israel, Korea, Australia and Greece (Figure 2). Despite the vast research, a practical solution to eradicate this parasite is yet to be formulated. This is the context which prompted us to highlight this issue by discussing the aspects including the diagnosis, immunological response and existed/proposed remedial measures against *C. irritans* in host fish.

2 | DIAGNOSIS OF EARLY INFECTION

Earlier, there was no appropriate molecular tool for the early detection of *C. irritans*-infected fish. Chen et al. (2008) developed first polymerase chain reaction (PCR) assay for the rapid and specific detection of *C. irritans* infection in marine fish followed by the standardization of quantitative PCR by Taniguchi in 2011 (Table 1). Recently, an enzyme-linked immunosorbent assay (ELISA) was also developed to diagnose the infected and asymptomatic fish using selected recombinant proteins as antigens (Lokanathan, Mohd-Adnan, Kua, & Nathan, 2016). An actin-depolymerizing factor gene (CiADF2) was cloned from *C. irritans*, and its transcripts were detected in all life cycle stages of the parasite. At the protein level, CiADF2 is abundant in the plasma around cytostomes suggesting its role in ciliate movement (Huang et al., 2013). This indicates the possibility to use this molecule (CiADF2) to diagnose the early parasitic infection (Figure 3). A reliable and commercial methodology to screen the *C. irritans* immediately after the infection is crucial for proper remedial action.

3 | RESEARCH ON IMMUNE RESPONSE IN HOST FISH

3.1 | Laboratory maintenance of *Cryptocaryon irritans*

As the systematic study on immunological response requires a constant supply of infective theronts, it is essential to maintain the parasite in the laboratory condition. Initially, the procedure was developed to culture the *C. irritans* up to 48 weeks (Burgess, 1992). Further modification in the procedure enabled the extension of the propagation period for up to 2 years (Yoshinaga & Dickerson, 1994). By using the host fish, *Trachinotus ovatus*, Dan, Li, Lin, Teng, and Zhu (2006) was successful to maintain *C. irritans* for 40 consecutive cycles for approximately 40 weeks. Yoshinaga et al. (2007) formulated an in vitro culture medium consisting of cultured fish cells (FHM) and agarose gel in order to raise *C. irritans* in vitro without the host fish. After a couple of years, Dan et al. (2009) developed the technique to preserve the *C. irritans* in vitro in their trophont and tomont life stages at a low temperature of 12°C for 4–5 months. According to the author, these dormant life stages (trophont and tomont) were able to produce not only the infective theronts but also generate progenies in the host fish. The said procedures could be adopted in the future studies rather than directly using host fish to maintain the *C. irritans*.

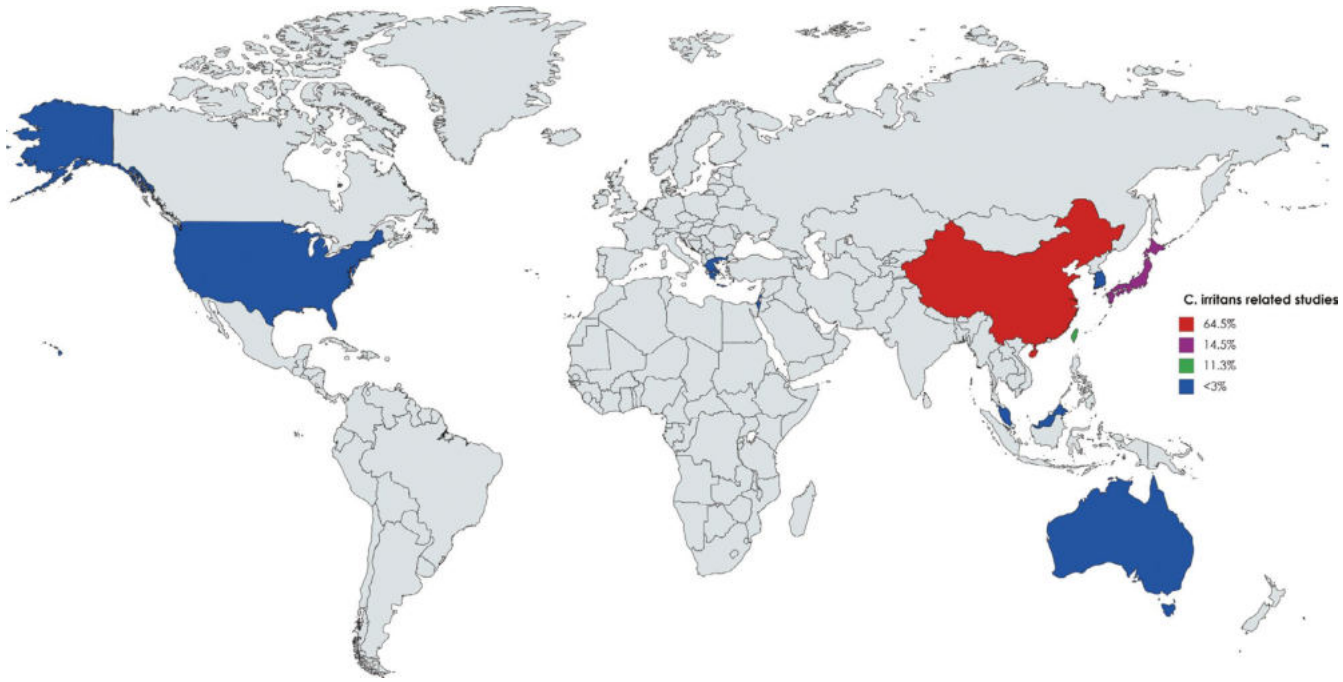

FIGURE 2 Percentage of research articles published from different countries all over the world [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Polymerase chain reaction (PCR) diagnosis of *Cryptocaryon irritans* infection

Primer name	Sequence (5′–3′)	Reference	PCR/primer sets	rDNA target sequence	Specificity
P1	GTTCCCCTTGAACGAGGAATTC	Yoshinaga & Dickerson, 1994	P1/NC2	Partial 18S, ITS1, 5.8S, ITS2 and partial 28S	Amplify rDNA from *C. irritans*
NC2	TTAGTTTCTTTTCCTCCGCT	Sun et al., 2006			
S15	TGAGAGAATTAATCATAATTTATAT	Chen et al., 2008	P1/S15	Partial 18S, ITS1, 5.8S and partial ITS2	Amplify rDNA from *C. irritans*
			Nested PCR PCR–1: P1/NC2 PCR–2: P1/S15		Amplify rDNA from infected water bodies
CI–120f	TGG CTC CCA TAA CGA TGA AGA	Taniguchi, Onishi, & Eguchi, 2011	CI–120f/ CI–120r	rDNA	Detect theronts from surface seawater
CI–120r	AAC ATG CCG TTG GGA TAT CC				

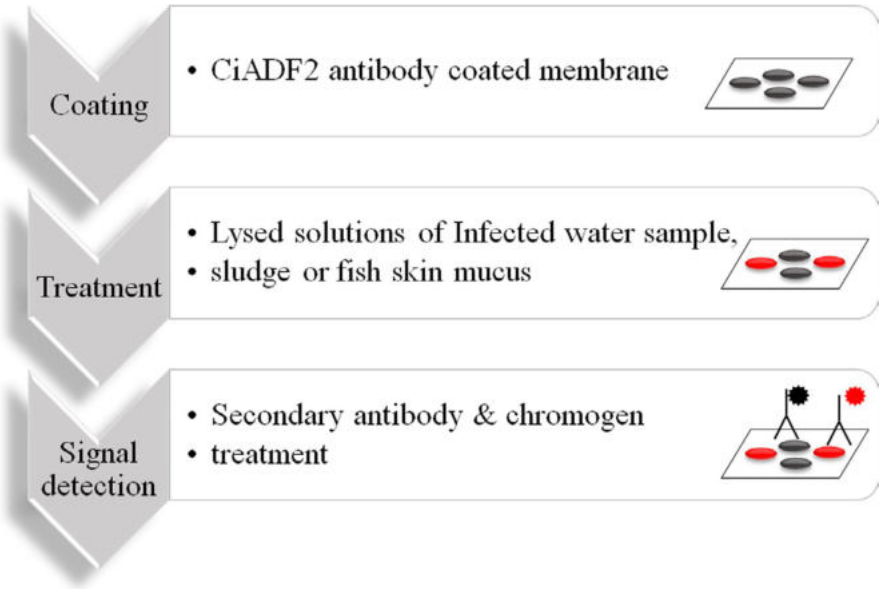

FIGURE 3 Diagnosis procedure proposed to detect early infection of *Cryptocaryon irritans* [Colour figure can be viewed at wileyonlinelibrary.com]

3.2 | Immune responses in host fish

In the immunological perspective, the first evidence of acquired response was witnessed in the mullet fish *Chelon labrosus* after natural exposure and intraperitoneal immunization with *C. irritans*. By using murine monoclonal antibodies raised against trophonts, two major polypeptides (20-21kDa and 68-69kDa) were recognized. *C. irritans*-specific antibodies, which were identified in the mullet serum, have caused parasite immobilization and agglutination in vitro (Burgess, 1992). In the further studies, immune protection in vivo was also confirmed in host fish once it is immunized with *C. irritans* (Burgess & Matthews, 1995a, 1995b; Yambot & Song, 2006; Yoshinaga & Nakazoe, 1997). Among the life cycle stages, theronts were identified as the most appropriate stage for immunization as it provides the strongest protective immunity evidenced from the comparative study on immunization in the host fish (*Epinephelus coioides*) with the homogenates of each stage (theront, trophont and tomont) (Bai, Xie, Zhu, Dan, & Li, 2008). Chemo-attraction assays followed by immunofluorescence and immunostaining using sera and mucus from infected fish enabled the investigators to identify the putative agglutination/immobilization antigen on the surface of *C. irritans* (Hatanaka, Umeda, Yamashita, & Hirazawa, 2007; Luo et al., 2008). A cDNA library of *C. irritans* tomonts was created, and the expressed sequence tags (ESTs) of transcriptome showed potential genes for the control of cryptocaryoniasis (Lokanathan, 2010). In continuation, eight highly expressed genes were chosen from the ESTs and attempt was made to express in *E. coli* after the stop codons were replaced with universal glutamine codons. The resultant recombinant proteins were proved to be efficient to detect the immunized and naturally infected fish sera (Lokanathan et al., 2016). If these recombinant proteins were tested for their effect on the fish survival, possible antigenic proteins could be identified. These preliminary studies proved the capability of acquired immune response developed in the host fish either by natural exposure or via other means of immunization with *C. irritans*.

When the susceptibility for *C. irritans* infection was tested among the eight cultured marine fish species from diverse families, except

rabbitfish (*Siganus oramin*), all tested species showed the infection with high intensity causing their massive mortality. Interestingly, the serum of *S. oramin* has been proved to have a strong killing effect on *C. irritans* in vitro as the fish serum induces the theront cilia fall-off, rupture of the cell membrane and the macronucleus. Subsequent biochemical experiments enabled to identify a novel antiparasitic protein (APP) from this host fish (*S. oramin*) serum, and MALDI-TOF-TOF-MS results revealed that this homogenous polymeric protein (61.7 kDa) is with an N-terminal amino acid sequence of SSVEKNLAACLRDND (L-amino acid oxidase). Laser confocal fluorescence microscopy confirmed that cell membrane and nucleus (of the parasite) are major site of APP action (Wang, Xie, & Li, 2010). Piscidins, another important component of the innate immune system, were found to have potent activity against *C. irritans* (Niu et al., 2013). Similarly, a recombinant antiparasitic protein (rSR-LAAO) expressed in *E coli* showed significant cytotoxic effect on *C. irritans* theronts (Li, Dan, & Li, 2013). More research pertaining to the application of these proteins as immune stimulants for escalating the innate host defence mechanism is highly warranted.

The expression patterns of IL-8, COX-2, C-type lectin and transferrin in orange-spotted grouper revealed that the local immune organs are more active against *C. irritans* than the systemic organs (Li, Dan, Zhang, Luo, & Li, 2011). In this report, an up-regulation of IL-8 in the infected fish skin suggests its possible role inducing chemotaxis of neutrophils and other granulocytes favouring their migration towards the site of infection; transferrin, which was up-regulated in the skin and gills of infected fish, may enhance the killing response of macrophages. Similarly, Cox-2 an inflammatory-related enzyme gene closely associated with the fish innate immune response was significantly up-regulated in the infected gill suggesting its role during the initial phases of *C. irritans* infection. Also, up-regulated expressions of C-type lectins and the Ca2+ dependent carbohydrate-recognition proteins in the *C. irritans*-infected fish skin and gills reveal the role of non-self-recognition. Further, the involvement of toll-like receptor (TLR) signalling pathway against *C. irritans* was revealed after significant changes in the expressions of TLR, MyD88 and IL-1β found within the skin, gill, head kidney and spleen (Li, 2012; Li, Luo, et al., 2011; Luo, Xie, Zhu, & Li, 2007). In the *C. irritans*-infected fish, TLR activation might have been associated with MyD88-dependent pathway leading to the production of the potent inflammatory cytokine IL-1β. The transcriptome analysis of liver from the infected host fish, *Lates calcarifer*, revealed a high-level expression of the genes encoding acute phase response genes such as hepcidin, C-type lectin and serum amyloid A (Khoo, Abdul-Murad, Kua, & Mohd-Adnan, 2012). The acute phase response rises as non-specific complex innate reaction shortly after tissue damage. The low expression levels of innate immune-related genes in the host fish recovered from *C. irritans* infection (approximately 10 days post-infection) suggesting that the innate immune system activated by the onset of parasite infection (2–3 days post-infection) would help the fish to recover (Khoo et al., 2012; Mohd-Shaharuddin, Mohd-Adnan, Kua, & Nathan, 2013). Expression of MHC class IIα gene was also modulated maximum followed by CC chemokine and hepcidin-2 precursor genes in liver and kidney of the *Lates calcarifer* (Mohd-Shaharuddin et al., 2013). MHC class II molecules play essential role in presenting peptide antigens to CD4+ T lymphocytes in the specific acquired immune system, while chemokines and hepcidin are involved in non-specific innate immune system depicting the participation of both innate and adaptive responses against *C. irritans*. In the systemic level, a transient up-regulation of macrophage colony-stimulating factor and its receptor (M-CSF/M-CSFR) followed by rapid down-regulation has also been reported in the head kidney of *C. irritans*-infected grouper (*Epinephelus coioides*) suggesting the role of M-CSF in the immune system of fish as in mammals (Dan, Zhong, Li, Luo, & Li, 2013). M-CSF can up-regulate the expression of chemokine receptor CXCR3 in head kidney macrophages and could traffic macrophages to the sites of infection where the CXCR3 ligands are expressed. Apart from these non-specific responses, acquired immune responses (including serum immobilization titre, blood leucocyte respiratory burst, serum alternative complement activity, serum lysozyme activity, IL-1β expression in spleen) have also been noticed in the host fish immunized with different doses of *C. irritans* theronts; the survival rate of the immunized fish was found immunization dose-dependent (Dan, Zhang, Li, & Li, 2013). More specifically, gene transcripts of tumour necrosis factor receptor (TNFR)-associated factor 6 (TRAF6), a crucial signal transducer in both the TNFR superfamily and toll-like receptor/interleukin 1R family, have also shown a marked increase in the immunized fish signifying its role against cryptocaryoniasis (Li et al., 2014). In another study, Mo, Chen, et al. (2015) identified two CCR6s (chemokine receptor 6) and both genes along with one of its ligand showed significant up-regulation in the skin of infected fish suggesting that chemokine receptors and their ligands may have important role in immune cells' homing to skin mucosal tissues during infection as in the case of mammalian system. Similarly, IL-34 and its receptor MCSFR2 identified in the host fish (grouper) were found to be strongly up-regulated followed by their down-regulation in the infected tissues indicated prolific activity of haemopoietic organ to supply the phagocytes to the site of infection (Mo, Li, et al., 2015). In a comparative gene transcription analysis using the transcripts of liver tissues from control and *C. irritans*-immunized large yellow croaker (*Larimichthys crocea*), a significant number of DEGs and isogenes (including those involved in major immune-related pathways such as toll-like receptor, complement and coagulation cascades and chemokine signalling pathways), as well as simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs), were identified (Wang et al., 2016). The transcriptome analysis carried out in the host fish in an infection dose-dependent manner demonstrated that low-concentration infection can significantly induce the complement and coagulation cascade pathway in host fish, while in higher concentration, the immunity status showed marked suppression (Yin, Gao, Tang, et al., 2016). The study based on the transcriptomes of the *C. irritans* tomonts under low temperature (by adopting the paired-end sequencing strategy and assembling the entire transcriptome de novo from three libraries such as tomonts at 12°C and 25°C and control), revealed the genes involved for tomont dormancy (Yin, Sun, Wang, & Gao, 2016). Another report based on the transcriptome analysis of piscidin-treated theronts and trophonts shows that most of the up-regulated genes in the treated ones are involved in cell migration and

TABLE 2 Serotype discrimination of *Cryptocaryon irritans*—experimental trials

Isolates	rDNA sequence	Agglutination/ immobilization		ELISA		Western blotting (antibodies)		Cross-protection	iAg phylogeny	Reference
		Serum	Mucus	Serum	Mucus	Serum	Mucus			
W1/K1	Identical	High	(-) ve	High	Low	(+) ve	(-) ve	Yes	ND	Ichiro Misumi et al., 2011
G32/ G37	Not identical	High	Low	ND	ND	ND	ND	ND	Different clade	Hatanaka, 2008; Lokanathan et al., 2016
cn39 cn40 cn41 cn42 Taiwan1 Taiwan2 G32−1,2,3,4,5	-	ND	ND	ND	ND	ND	ND	ND	Clade I	Lokanathan et al., 2016
cn100/ cn101/ cn102/ G37		ND	ND	ND	ND	ND	ND	ND	Clade II	
cn56/ cn57		ND	ND	ND	ND	ND	ND	ND	Separate clade	

Note: ND: not determined

apoptosis (Chen et al., 2018). All these transcriptome studies reveal the potentiality of major host genes involved in parasitic resistance and dosage of immunization as well as the important genes of *C. irritans* involved in parasite life span.

In addition, up-regulations of IgT and IgM (in BCR signalling pathway) have also been noticed in the head kidney and spleen of the infected host fish (grouper) signifying the role of B cells in the systemic tissues. Apart from this, the up-regulated expression of protein kinase genes in the skin suggested that B cells may also be activated at the local infection site (Mo et al., 2016). XCR1, the unique receptor of the XC subfamily of chemokines, was found up-regulated in the skin and the spleen of *C. irritans*-infected grouper revealing its vital role in regulating the localization and function of T cells, dendritic cells and other cell types (Ni et al., 2017). In an immunoproteomics study, a total of 12 proteins differentially expressed among the three life cycle stages of *C. irritans* were identified, and out of which 9 proteins elicited serological responses in rabbits and 10 antigenic proteins were able to react with fish anti-sera (Mai et al., 2015). These findings indicate the scope for developing the tool for remedial measures (for instance, vaccine) against *C. irritans*.

4 | CONTROL MEASURES

4.1 | Conventional approach

Following the general and customary eradication methods stated (Dickerson, 1994), different treatment strategies have been tested against *C. irritans*. Hypersalinity, quinine hydrochloride, chloroquine and minimal handling were among the effective treatment regime against recurring *Cryptocaryon* infections (Huff & Burns, 1981; Picón-Camacho, De Ybáñez, Holzer, Arizcun, & Muñoz, 2011). The defensive effects of bovine lactoferrin (Kakuta & Kurokura, 1995), medium-chain fatty acids (Hirazawa, Oshima, Hara, Mitsuboshi, & Hata, 2001), caprylic acid (Hirazawa, Oshima, & Hata, 2001), in-feed inhibitors of folic acid synthesis and dihydrofolate reductase (Kawano & Hirazawa, 2012a), dietary Romet®30 (sulfadimethoxine–ormetoprim (SDMX–OMP)) (Kawano, Hirazawa, Gravningen, & Berntsen, 2012b), dietary chromium polynicotinate (Wang, Ai, Mai, Xu, & Zuo, 2014), herbal extracts (Goto, Hirazawa, Takaishi, & Kashiwada, 2015a), matrine and oxymatrine (Goto, Hirazawa, Takaishi, & Kashiwada, 2015b), and leptomycin B (Yin, Sun, Tang, Gong, et al., 2016) were investigated for their potential to inhibit cilia-based motility of, *Cryptocaryon irritans*, during its infective (theront) phase. Other mechanical control measures included interrupting the life cycle of *C. irritans* via the removal of its tomonts (Jiang et al., 2016), treatments based on betadine, formalin, freshwater, malachite green, oxytetracycline, 2-phenoxyethanol, potassium permanganate and trichlorphon (Pironet & Jones, 2000) and nitazoxanide immersion (Fan, Lin, Zhong, & Qin, 2016). Quinine, a potential antiparasitic compound, was also found to be significant to reduce the intensity of infection, particularly when the fish are lightly infected (Rigos et al., 2013). Another recent study by Hyun Kim, Fridman, Borochov-Neori, Sinai, and Zilberg (2019) revealed that garlic extract which contain allicin, the main active ingredient, could completely immobilize the theronts and protomonts within 20- to 40-min duration, and in vivo treatment trials in *Poecilia reticulate* resulted a reduction in infection intensity on the caudal fin. In another in vitro experiment, both the parasitic trophont and encysted tomont of *C. irritans* showed normal developments at 25, 28, and 31°C, while their development was badly

damaged at 34°C. It also showed that hypoxic (24%) condition is able to maintain the tomonts at dormant state, and at hyperoxic (141%) and anoxic (0%) conditions, no resurgence is possible (Yoshinaga, 2001). In another experiment, exposure to heat (40°C) and chlorine (more than 60 ppm) prevented the hatching of their cysts or destroyed the hatched theronts (Hirazawa, Goto, & Shirasu, 2003). In a recent report, theronts were found dead and tomonts were in the state of quiescent after exposure to anaerobic condition in the intensive recirculating aquaculture systems (RASs) (Standing et al., 2017). All these reports well indicate the possibility of the development of effective control measures in aquaculture like keeping the physical conditions unfavourable to the parasite at least for a short period to avoid their outbreak but without affecting the viability of the fish under culture system.

4.2 | Immunological approach

Detection of specific antibodies in the serum and skin mucus of host fish upon the surface exposure or intraperitoneal injection of theronts indicates sign of acquired humoral and mucosal immunity against *C. irritans* thanks to the work by Luo et al. (2007). The immune protection was also noticed in the immunized fish, and the optimized immunization dose was reported to be 10 theronts/g fish BW (Misumi, Leong, Takemura, & Lewis, 2012).

4.3 | Molecular approach

An immobilization antigen (iAg), 34 kDa, was characterized by Bai et al. (2008), and a full-length cDNA of iAg was characterized by Hatanaka (2008). iAg contains 1171 base pairs encoding a protein of 331 amino acids with hydrophobic N-terminal and C-terminal glycosylphosphatidylinositol anchor (Hatanaka, 2008). The antigenic property of iAg against theronts was confirmed through immobilization activity of iAg-immunized fish serum. Based on the laboratory experiment results showing the expression of different iAg, Hatanaka et al. (2008) proposed the possibility of more serotypes of *C. irritans*.

According to their studies, serotype G32 caused only little immobilization towards the theronts of serotype G37 and vice versa, and also, ITS rDNA sequence of G32 was not found identical to that of G37 (Hatanaka et al., 2008). Though this in vitro observation was supported by the further studies (Misumi, Lewis, Takemura, & Leong, 2011), in the in vivo experiment, two different *C. irritans* isolates based on rDNA analysis provided cross-protection among themselves (Misumi et al., 2011) (Table 2). In an another study by Lokanathan et al. (2016), the phylogenetic data derived from iAg amino acid sequences of 9 isolates of *C. irritans* revealed only three clusters of iAg variants presumably the three serotypes. From these observations, we were tempted to suggest that Western blot analysis of serum antibodies against crude proteins of the different C. *irritans* isolates would have been helpful to discriminate the serotypes more or less precisely apart from rDNA sequencing, in vitro immobilization and ELISA.

Huang et al. (2012) characterized the cDNA of an iAg named as CiSA-32.6 (GenBank ID: JF812643), and they were also successful to express its protein in the bacterial cells after sequence modifications (such as removing the signal peptide and hydrophobic C-terminal as well as by modifying the non-universal genetic codes) and developed the *C. irritans*-specific antibodies.

TABLE 3 Potential vaccine candidates and their immune responses based on the literature

Immune responses of fish immunized with *C. irritans*				Vaccine candidate	Reference
Local (skin and mucus)	Adaptive	humoral	Mucus iAg-specific IgM	iAg	Josepriya et al., 2015
		cellular	$CD8^+$ skin leucocytes	iAg	
	Innate	humoral		Cn10, cn48, cn72, cn110	Lokanathan et al., 2016
Systemic (spleen and circulation)	Adaptive	humoral	IgM_H	iAg cn56, cn57	Josepriya et al., 2015; Lokanathan et al., 2016
			IgT_H	iAg	Josepriya et al., 2015
		cellular	MHC I		
			CD4		
			CD8α		
	Innate	humoral	IFNγ		
			TNFα		
			IL12p40		
		cellular	MHC II	*C. irritans*' theronts	Dan, Zhang, et al., 2013
			Il−1β		
			IL−11	*C. irritans*' theronts	Wu et al., 2019

Significantly, Jose Priya et al. (2012) could develop a potent DNA vaccine against *C. irritans* for the first time by modifying the codons of the iAg (by changing the stop codons with appropriate one without affecting the deduced three-dimensional structure of the protein). They were also successful to optimize the iAg vaccine for higher protection by adding the DNA sequences of fish immunoglobin M signal peptide and heat shock protein C-terminal domain as adjuvant (Josepriya et al., 2015). Parallel to this work, interleukin-12 (IL-12) was also proved as a plausible molecular adjuvant for the iAg vaccine (Tsai et al., 2014). Apart from this, two other promising vaccine candidates such as CiADF2 and Ci14-3-3 were also recommended by Huang et al. (2013) and Lin et al. (2013). CiADF2 was proved for its effective role in the ciliate movement of *C. irritans* theronts (Huang et al., 2013). Since Ci14-3-3 is expressed at all life stages of *C. irritans* and its protein is present in cytoplasm, plasma membrane and the front end of cytostome in newly hatched theronts, and also by considering the capability of its antibodies to cause immobilization of theronts, it may be considered as a potential vaccine (Lin et al., 2013). Similarly, anti-*C. irritans* active substances in the skin mucus could also able to stop swimming and cilia movement of C. *irritans* (Lin et al., 2013). In Table 3, potential vaccine candidates for *C. irritans* infection are listed.

5 | SUMMARY AND CONCLUSIONS

Over 60% of the published reports on *C. irritans* were from China, the world's largest fish-farming country. Despite the vast literature on the immune responses of various fish hosts against *C. irritans*, the data are not sufficient to deduce a specific disease control measure. Though qPCR assay was standardized for the detection of *C. irritans*, relying this technique to detect the early infection is not satisfactory. As a solution to this problem, an actin-depolymerizing factor (CiADF2) responsible for the cilia movement could be considered as the screening protein as it is expressed only during the initial stages of infection. Conventionally, culture temperature of 31°C to 34°C, exposure to heat and chlorine, hypoxic, hyperoxic or anoxic dissolved oxygen content at the water bottom, anaerobic conditions of intensive RASs, caprylic acid, quinine and allicin have been proved effective against intensive *C. irritans* infection. For the disease-prone culture ponds, immunization with infectious theronts is suggested to be the most effective method. However, continuous collection of theronts needs laboratory maintenance practice. Cryopreservation technique proposed for trophonts and tomonts of *C. irritans* could be optimized in such a way to preserve them with maximum output. Despite immunization by iAg yielded significant anti-*C. irritans* effect, it is not equally effective against its different isolates particularly for different serotypes. This necessitates to identify the specific molecular regions of iAg protein for the serological discrimination of *C. irritans*. Apart from iAg, CiADF2 and Ci14-3-3 were proposed as promising vaccine candidates. Application of interleukins and other cytokines may be useful as molecular adjuvants. The degree of response triggered by skin and gill mucosal components of the host fish against *C. irritans* is significantly high compared with that provided by the systemic blood components indicating the predominant role of innate immunity. Up-regulated expressions of IL-8, COX-2, C-type lectin, transferrin, TLR, MyD88, IL-1β, CCR6, M-CSF receptor and its alternative ligand IL-34, IL-34 and its receptor EcMCSFR2, protein kinase genes and XCR1 found within the skin and gill after *C. irritans* infection also support the active participation of innate immunity against the *C. irritans*. The role of systemic blood for both innate and acquired immune response against this parasite has also been confirmed. The active presence of L-amino acid oxidase, hepcidin, C-type lectin, serum amyloid A, MHC class IIα and piscidins in the infected fish blood signifies the role of systemic innate immune response. On the other hand, the adaptive immune response is also evidenced by the presence of *C. irritans*-specific IgM and IgT antibodies.

From the overall studies on host fish immune responses against *C. irritans* infection, it is concluded that despite the diverse level of immune responses is in place, the local innate cellular immunity dominates with immediate response. The picture is still incomplete. More research is recommended on the following line. Apart from the iAg, other possible antigen candidates such as CiADF2 and Ci14-3-3 need to be explored. Experimental confirmation is mandatory to check the role of innate responsive components as genetic adjuvants, if any. These studies would be immensely helpful to develop effective vaccines against *C. irritans* infection and thereby optimize the aquaculture practices.

ETHICS STATEMENT

The manuscript did not use any specimen in the listed category of experimental animals which need ethics approval.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

TAJP analysed the data and drafted the paper. SK critically reviewed the paper.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

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