An update on the detection methods of *Parachlamydia acanthamoebae*, an atypical agent of pneumonia

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Abstract. *Parachlamydia acanthamoebae* (*P. acanthamoebae*) has been recognized as an emerging agent of pneumonia as it has been identified in human samples via culture-based, molecular and serological techniques. Additionally, studies on animal models have shown that it fulfills the third and fourth Koch postulates to be assigned a pathogenic role. Due to the threat posed by it, multiple tools have been employed in the search for *P. acanthamoebae*. The methods utilized for its detection would be cell culture based approaches which involve both animal and amoebal cell culture and also molecular techniques that encompasses polymerase chain reaction (PCR), fluorescence in situ hybridization (FISH) and in situ hybridization (ISH). Additionally, immunohistochemistry (IHC) and serology based techniques such as direct and indirect immunofluorescence are also employed with the usage of Western blotting or immunoblotting as confirmatory procedures. This review attempts to describe the variety of techniques that are present in literature for the isolation and identification of *P. acanthamoebae*.

Keywords: Atypical agent, culture-based, detection, molecular techniques, pneumonia, serology

INTRODUCTION

The origins of the word pneumonia can be traced back to the ancient Greek where the word “pneumon” means “lung” and therefore the word pneumonia means “lung disease” (Sattar and Sharma, 2018). Pneumonia is defined as an infection of one or both sides of the lungs which leads to the alveoli of the lungs to be filled with pus (Pneumonia | National Heart, Lung, and Blood Institute (NHLBI), 2018). The morbidity and mortality contributed by the condition is high as it kills about 2,400 children a day aged under five, with a total death toll of about 880,000 children in 2016 (Pneumonia in Children Under Five- UNICEF DATA, 2018). A search of literature would reveal that there are multiple etiologic agents commonly ascribed to be causes

of the condition (Figure 1). Due to the ubiquity of these agents and the fact that a large portion of cases cannot have an etiology pinpointed, it is common to overlook organisms which are not traditionally noted for causing pneumonia. *Parachlamydia acanthamoebae* (*P. acanthamoebae*) is one such example. *P. acanthamoebae* is a Chlamydia-like organism which is obligately intracellular that has been noted to infect free-living *Acanthamoeba* (Hayashi *et al.*, 2010). It can stain either Gram positive or Gram negative depending on its infectious stage (Amann *et al.*, 1997). The life cycle of *P. acanthamoebae* in *Acanthamoeba polyphaga* (*A. polyphaga*) is well documented and it has been demonstrated that there are three varying development stages for the said pathogen which

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would be the reticulate, elementary and crescent bodies (Amann *et al.*, 1997; Greub and Raoult, 2002a). The various developmental stages of *P. acanthamoebae* have been identified to be distributed in varying compartments of the amoebal cell yet all of them have a similarity whereby they are commonly visualized in the vacuoles of the amoeba (Greub and Raoult, 2002a). However, reticulate bodies are predominantly found in the cytoplasm of the host while the crescent bodies appear to be occasionally present in close proximity of the host. The infective stages of *P. acanthamoebae* have been identified to be the elementary and crescent bodies and their intake is mediated by phagocytic action of the amoebal host cell (Greub and Raoult, 2002a). Due to the ubiquity of amoeba in

the environment, it can be postulated that they may act as a reservoir or a vector of sorts for *P. acanthamoebae*. Indeed, *P. acanthamoebae* has been identified in *Acanthamoeba mauritaniensis* (*A. mauritaniensis*) and *Acanthamoeba castellani* (*A. castellani*) which were isolated from the nasal mucosa of healthy volunteers (Amann *et al.*, 1997). Additionally, the presence of *P. acanthamoebae* has been noted in bovines, ruminants, reptiles, boars and even guinea pigs (Borel *et al.*, 2007; Di Francesco *et al.*, 2013; Lutz-Wohlgröth *et al.*, 2006; Ruhl *et al.*, 2008; Soldati *et al.*, 2004). As such, it is possible that these animals could be efficient vectors or reservoirs as some of them are kept as pets by people while the remaining fall under the livestock category.

Figure 1. Distribution of various etiologic agents of CAP (N=13673) on the basis of review of literature, 2009-2019. Abbreviated pathogens are *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Mycoplasma pneumoniae*, *Legionella pneumophila*, *Moraxella catarrhalis*, *Chlamydia pneumoniae* and *Coxiella burnetii*. (Akter *et al.*, 2014; Aliberti *et al.*, 2013; Caggiano *et al.*, 2017; Capelastegui *et al.*, 2012; Cillóniz *et al.*, 2011; Farida *et al.*, 2015; Holter *et al.*, 2015; Huijskens *et al.*, 2013; Jain *et al.*, 2015; Köksal *et al.*, 2010; Luchsinger *et al.*, 2013; Lupisan *et al.*, 2019; Mermond *et al.*, 2010; Naik *et al.*, 2010; Nascimento-Carvalho *et al.*, 2016; Quah *et al.*, 2018; Shibli *et al.*, 2010; Spoorenberg *et al.*, 2014; van Gageldonk-Lafeber *et al.*, 2013).

Previous research is strongly suggestive of the notion that *P. acanthamoebae* is an emerging pathogen. The first instance of this would be the identification of Hall's coccus in an amoeba which was isolated from a case of humidifier-associated fever (Birtles *et al.*, 1997). A study done on community-acquired pneumonia (CAP) patients in Nova Scotia, Canada, revealed a seroconversion rate of 2.2% towards *P. acanthamoebae* whereby 8 out of 371 patients had antibodies which were directed against it compared to 0 out of 511 healthy volunteers (Marrie *et al.*, 2001). The data garnered suggests that it is not a normal component of the human microbiome, however literature search purports that it is likely an opportunistic pathogen of the respiratory system. Indeed, *P. acanthamoebae* was detected in an individual that was under immunosuppressants after receiving an allograft (Marrie *et al.*, 2001). Additionally, it was also identified in a human immunodeficiency virus (HIV)-positive patient that only had 80 CD4 cells/mm³ (Greub, Berger, *et al.*, 2003). The stance is supported further by a study from Greub and coworkers which identified notable antibody titers against *P. acanthamoebae* in 5 out of 37 (13.5%) polytraumatized intensive care unit patients (Greub, Boyadjiev, *et al.*, 2003). Its medical burden has also been noted in children, which are a population group that do not have completely developed immune systems (Lamoth *et al.*, 2011). The success of *P. acanthamoebae* as an opportunistic respiratory pathogen may lie in its temperature dependent lytic activity. The pathogen has been noted to cause the lysis of *A. polyphaga* at temperatures between 32-37°C while remaining an endosymbiont at 25-30°C (Greub, La Scola, *et al.*, 2003). Therefore, it is proposed that the bacteria employ amoeba as a reservoir while in the surrounding environment and is liberated via lysis of the amoeba once it is at a higher temperature such as that in the respiratory tract. The concern raised by the pathogen is worrisome as it has been detected via molecular methods in the hospital environment itself (Fukumoto *et al.*, 2010).

The isolation of *P. acanthamoebae* is a laborious process as the pathogen is obligately intracellular and therefore the conventional "gold standard" culture methods will not yield results. The fastidious intracellular pathogen is only

cultivable from clinical samples via the usage of amoebal co-culture techniques which are not routinely performed in diagnostic laboratories (Greub, 2009). Adding to the conundrum, would be the absence of specific tests or diagnostic kits to identify its presence in a sample. The amount of detection techniques present for it are chiefly molecular based methods and these are also not commonly done for *P. acanthamoebae*. Due to the delay in identifying the pathogen, the administration of treatment is also impeded hence contributing to further decline in the patient's health. Within this context, this review attempts to describe the variety of techniques that are present in literature for the isolation and identification of *P. acanthamoebae*.

1. Culture based methods

The utilization of culture based techniques for the purpose of identification is rather time consuming, yet it has the benefit of permitting the recovery of actual strains of the pathogen hence adding to the knowledge pool available pertaining it. Additionally, it removes the element of doubt from a diagnosis whereby the existence of viable organisms can be illustrated by the propagation of the said organism *in vitro*. A search of literature would reveal that in the context of *P. acanthamoebae*, there are two different types of culture based methods for pathogen identification.

1.1 Amoebal co-culture

This system can be described as a variant of conventional cell culture whereby instead of normal cells, amoeba are employed as the cells (Greub, 2009). The common amoeba used in this strategy are *Acanthamoeba* spp., as they have been demonstrated to be the natural hosts for multiple amoeba-resisting bacteria (Corsaro and Greub, 2006). In utilizing *Acanthamoeba* spp., for the isolation of *P. acanthamoebae*, *A. castellanii* appears to be the most commonly employed species yet it should be noted that there is a paucity of literature which recommends the usage of a specific species. As such, it is suggested that multiple species of *Acanthamoeba* and/or different genus of amoeba should be considered as it may enhance recovery rates of *P. acanthamoebae*. The amoebal co-culture

strategy can be divided into two different types which would be the direct amoebal co-culture and the amoebal enrichment method. In direct amoebal co-culture, amoebae are used as the cell background and clinical samples are added in to isolate out the *P. acanthamoebae* that may be present in the sample (Greub and Raoult, 2002b). In amoebal enrichment, the goal is to recover amoebae that are free-living. This is done via addition of the clinical sample to non-nutrient agar which is supplemented with either living *Escherichia coli* (*E. coli*) or *Enterobacter cloacae*, followed by an incubation at 25-30°C and daily observation for amoebal presence which should then be examined for the presence of *P. acanthamoebae* (Greub and Raoult, 2002b). In a clinical setting the former could be possibly more effective as it may allow the direct isolation of *P. acanthamoebae* from samples while the latter could have higher efficacy in infections which are polymicrobial in nature as it will allow the amoebae to be isolated out hence permitting the recovery of *P. acanthamoebae*.

1.2 Cell culture

Prior research suggests that cell cultures are generally unsuccessful in recovering *P. acanthamoebae* from samples. A study by Hayashi and colleagues revealed that no growth of the pathogen was observed in HEp-2, Vero, Jurkat and THP-1 cells (Hayashi *et al.*, 2010). It can be postulated that the absence of growth was due to a loss of receptors or intake systems in mammalian cells which have adapted to stable culture environments. The finding by Hayashi *et al.*, is contradicted by Yamane and coworkers as they discovered that growth in Hep-2 cells is indeed possible at temperatures lower than the normal human body temperature albeit exhibiting low infectious rates (Yamane *et al.*, 2015). Adding to the conundrum, data from another group identified that *P. acanthamoebae* is able to enter and multiply within human macrophages in addition to causing apoptosis in the immune cells (Greub, Mege, *et al.*, 2003). This could probably be a survival mechanism of the pathogen whereby macrophages are utilized as a temporary replicative niche prior attachment to cells that are more suited for its growth. Indeed, it has been illustrated that *P. acanthamoebae* has the capability

to enter and multiply within pneumocytes and fibroblasts yet the viability of these cells is not compromised post-infection (Casson *et al.*, 2006). This data is suggestive that these cells may just be a temporary intrapulmonary niche for the strictly intracellular pathogen prior to exhibiting virulence or infecting better targets. It is rather vivid that the efficacy of mammalian cell culture for isolating *P. acanthamoebae* is questionable and as such alternatives should be explored.

Interestingly, the academic community has also conducted research on the use of cell lines from fish and insects for permissivity towards *P. acanthamoebae*. Work done on cell lines from *Aedes albopictus* (Asian tiger mosquito), *Spodoptera frugiperda* (fall armyworm) and *Drosophila melanogaster* (common fruit fly) demonstrated that *P. acanthamoebae* is able to replicate in the said cells albeit inducing severe cytotoxic effects in them (Sixt *et al.*, 2012). This stance is challenged by another group which highlights that *P. acanthamoebae* is not able to grow in cells from the Asian tiger mosquito and fall armyworm (Kebbi-Beghdadi *et al.*, 2015). It is possible that the cells may have just taken varying times to adapt to the varying culture conditions hence causing the discrepancy. In the context of fish cell lines, the permissivity of cells from *Pimephales promelas* (fathead minnow) and *Oncorhynchus mykiss* (rainbow trout) towards *P. acanthamoebae* has been identified to be unsatisfactory (Kebbi-Beghdadi *et al.*, 2011). The data obtained is suggestive that fish and insects are either niches for differentiation or a reservoir before infecting organisms that are better suited. Nevertheless, more work should be done on insect cell lines as it has the potential to be a new arsenal in the tools available for the diagnosis of *P. acanthamoebae*. However, the feasibility is questionable as well, as having insect cell lines for the diagnosis of a single pathogen does not seem economically rewarding for diagnostic laboratories.

2. Molecular Techniques

Molecular methods are commonplace in clinical microbiology as they are utilized to detect and/or identify the presence of microbes in clinical samples (Corsaro and Greub, 2006). These techniques are rather rewarding in the clinical

setting as they permit the diagnosis/detection of pathogens that may be fastidious.

2.1 Polymerase chain reaction (PCR) method

PCR is a process which consists of three sequential steps that are denaturation, annealing and extension (Ghannam and Varacallo, 2019). Denaturation occurs when the sample is heated up until about 95°C which causes disruption of the hydrogen bonds that hold the two strands of DNA template together. The sample then has its temperature reduced to about 50-65°C depending on the primer employed which then permits annealing of complementary base pairs. The final step, extension occurs at about 75-80°C which permits the DNA polymerase to synthesize new DNA strands that would be complementary to the template DNA strand.

The literature pertaining to the usage of PCR in the diagnosis of *P. acanthamoebae* suggests that the 16S ribosomal RNA (rRNA) is a commonly targeted region for amplification. This is not a surprise as targeting this gene is phylogenetically informative and as such will permit the identification of new strains or species. Casson *et al.* (2008) report a real time-PCR that target the 16S rDNA-encoding gene which exhibits high sensitivity and specificity. The sensitivity was just 10 copies of genomic DNA/μL while the specificity was illustrated via testing with genomic DNA of humans, fungi and bacteria which all yielded negative results (Casson, Posfay-Barbe, *et al.*, 2008). Similar PCR based methods targeting the 16S rDNA for amplification are reported elsewhere (Amann *et al.*, 1997; Fukumoto *et al.*, 2010; Le Faou *et al.*, 2001). Despite the aforementioned efficacy in the usage of 16S rDNA as a target for PCR amplification, there are some issues surrounding its employment. Indeed, multiple authors have managed to amplify samples of pathogens that have been isolated (Le Faou *et al.*, 2001; Wheelhouse *et al.*, 2010, 2011). However, subsequent analysis via bioinformatics commonly revealed that the pathogens are only from a certain genus or that the organisms identified have a certain percentage of sequence similarity to an existing pathogen (Wheelhouse *et al.*, 2010, 2011). As such, the discriminatory power and specificity can be questionable at times. Consequently, a positive PCR result obtained via

the use of these primers may not necessarily mean that *P. acanthamoebae* has been detected but rather it could indicate the identification of a new species or strain. On a positive light, it does add to the pool of knowledge present pertaining a pathogen yet it may not hold much value in a clinical setting.

There is a paucity of literature pertaining the use of genes other than the 16S rRNA. A study by Soldati and collaborators describes the use of 23S rDNA which was Chlamydiales order-specific to detect *P. acanthamoebae* (Soldati *et al.*, 2004). The study revealed that the sensitivity was better than culture based techniques employed. However, the length of the fragment generated via the PCR was rather limited and hence did not permit the conduction of phylogenetic analysis and comparative analysis. Another gene of interest for PCR amplification to detect *P. acanthamoebae* is the *tlc* gene. It encodes the nonmitochondrial ADP/ATP translocase that is involved in the exchange of bacterial ADP for ATP from the host cell hence permitting energy parasitism (Greub and Raoult, 2003). Work by Greub and coworkers utilized this gene as a target for PCR on bronchoalveolar lavage samples and reported that it was indeed effective albeit having false-positives (Greub, Berger, *et al.*, 2003). A theory proposed to explain the false negative would be the gene used for the amplification itself. Indeed, the *tlc* gene is present in *Rickettsiales*, *Chlamydiales*, plant and alga plastids hence it may lead to cross-amplification which would result in false-positives (Greub and Raoult, 2003). However the gene is still *sui generis* in bacteria as the bacterial version of the *tlc* gene product is absent of a N-terminal peptide transit of approximately 90 and 150 amino acids which is only present in green plants and alga plastids (Greub and Raoult, 2003). Due to this variance, it does add a certain amount of discriminatory power for the PCR assays which target this gene for the detection of *P. acanthamoebae*.

Collectively, existing literature demonstrates that previous authors have emphasized much on utilizing the 16S rRNA encoding gene as the PCR amplification target. The 23S rRNA encoding gene and *tlc* gene are merely being supplemental in nature when compared with the work done on the previous gene sequence. The data presented warrants that additional research should be conducted on optimizing the sequences employed during use of the 16S rRNA encoding gene as

there appears to be a certain lack of discriminatory power as reported by authors. The 16S rRNA gene has been noted to be omnipresent in all prokaryotes and it contains both fast-evolving and slow-evolving regions (Sambo *et al.*, 2018). As such, it is possible to design broad-spectrum primer pairs targeting the slow-evolving regions to obtain a PCR product and subsequently sequence information from the generated amplicon can be utilized to isolate out the species-specific fast-evolving regions hence contributing to an overall increase in discriminatory power (Sambo *et al.*, 2018). The key challenge of using the 23S rRNA encoding gene would be the dearth of established broad-range bacterial PCR amplification and sequencing primers and hence the solution would be to develop novel PCR assays targeting the said gene (Hunt *et al.*, 2006). In line with this, effort should also be placed in the direction of identifying new gene sequences that would permit the development of superior PCR based assays for the detection of *P. acanthamoebae*. It is suggested that PCR assays which target the internal transcribed spacer (ITS) region that links the 16S and 23S regions should be developed. Indeed, this has been employed before to detect and discriminate between various organisms such as *Lactococcus garvieae*, *Streptococcus iniae*, *Vibrio* spp., *Campylobacter* spp., *Legionella* spp, and *Clostridium difficile* (Berridge *et al.*, 1998; Hoffmann *et al.*, 2010; Mendoza *et al.*, 1998; Riffard *et al.*, 1998; Stubbs *et al.*, 1999; Thanh *et al.*, 2013). Targeting the ITS region for PCR has the inherent advantage of permitting intraspecies differentiation as species-species sequence polymorphisms can be detected easily because the ITS region varies greatly in size and sequence among closely related bacterial species in comparison with the more evolutionarily constrained 16S and 23S rRNA genes (Berridge *et al.*, 1998; Hoffmann *et al.*, 2010; Riffard *et al.*, 1998). Additionally, bacteria may have multiple alleles of the rRNA gene cluster and as such this would permit the differentiation of epidemiologically related and unrelated strains in addition to potentially increasing the sensitivity of the PCR assay (Kiss *et al.*, 1977; Thanh *et al.*, 2013). In the context of *P. acanthamoebae*, there is only one PCR assay available which targets the ITS region and even then the primers targeting the region were *Chlamydiaceae* order specific hence indicating

that this avenue has not been widely explored yet (Lutz-Wohlgroth *et al.*, 2006).

2.2 Fluorescence in situ hybridization (FISH)

The primary step in FISH would be to prepare probes which would be complementary to the gene sequence that one would be searching for (Ratan *et al.*, 2017). Labeling of the DNA probe can be done either directly or indirectly. In the former, probes are directly labeled with nucleotides containing a fluorophore while in the latter, the modified nucleotides containing a hapten would be labeled. However, for indirect labeling an extra step would be required to permit visualization of non-fluorescent hapten and this requires either an enzymatic or immunological detection system (Ratan *et al.*, 2017; Speicher and Carter, 2005). Post-labeling, both the labeled probe(s) and the target DNA are denatured to yield single-stranded DNA and then these are both combined hence permitting the annealing of complementary DNA sequences.

The efficacy of this technique to detect and identify microbes without any form of cultivation has been noted previously (Fritsche *et al.*, 1999; Horn *et al.*, 1999). In a clinical setting, this translates into a reduction of time required for diagnosis hence allowing prompt treatment of the patient. Additionally, FISH is commonly employed to analyze microbial communities in the context of composition and structure (Juretschko *et al.*, 1998; Snaidr *et al.*, 1997). This would be of high value in a diagnostic setting, as it could aid to discriminate *P. acanthamoebae* specifically if the infection in a patient is polymicrobial in nature. There exists a considerable body of literature pertaining the use of FISH for the detection of *P. acanthamoebae* (Amann *et al.*, 1997; Hayashi *et al.*, 2010; Matsuo *et al.*, 2008). However, all the reports illustrate usage of FISH for *P. acanthamoebae* detection in *Acanthamoeba* spp. with the exception being a study by Hayashi *et al.* (2010) which describes detection in mammalian cells as well. Interestingly, another group utilized the precursor to FISH which was in situ hybridization (ISH) to detect *P. acanthamoebae* (Amann *et al.*, 1997). The labeling was done with tetramethylrhodamine and horseradish peroxidase which is different from the usage of fluorescent probes employed in FISH. Their

study demonstrated a cross-reaction between two different strains of *P. acanthamoebae* and this suggests that FISH would be a technique which has very little use in the context of phylogenetics.

Existing literature purports that FISH is a valuable technique to detect *P. acanthamoebae* due to its rapidity and specificity. It has also been used to identify genotypic antibiotic resistance in *Helicobacter pylori* towards clarithromycin hence indicating that such an avenue could exist for *P. acanthamoebae* as well (Yilmaz and Demiray, 2007). In light of reported works pertaining use of FISH in detecting *P. acanthamoebae*, it can be deduced that there is not much effort in this direction. This is indeed surprising as FISH has the potential to be an imperative diagnostic tool of *P. acanthamoebae* granted its aforementioned advantages. It is also very cost-effective as it has been reported that the total cost for materials in a FISH reaction is less than 1 euro (Frickmann *et al.*, 2017). Accordingly, it is suggested that commercialization of FISH for the detection of *P. acanthamoebae* should be considered as the key obstacle is a lack of the techniques' usage in diagnostic laboratories (Frickmann *et al.*, 2017). However, this could be argued against as well because designing probes for the detection of a pathogen that is an atypical agent of pneumonia may not be economically profitable in the long run for diagnostic laboratories. Valorization is possible by introducing FISH assays which would be functioning to detect a panel of intracellular pathogens that are etiologic agents of pneumonia and within this panel FISH probes that test for the presence of *P. acanthamoebae* can be included as well.

3. Immunohistochemistry

IHC is a technique which is employed to detect specific antigens that may be present in a tissue section via the usage of antibodies where they are then visualized by using enzymes which would react with a specific substrate hence resulting in color change. In the context of clinical microbiology, IHC has the advantage of being usable in patients that are awaiting culture results, having low bacterial loads and partially treated (Kundu *et al.*, 2014). Additionally, it adds discriminatory power as the presence of *P. acanthamoebae* inside infected tissue can be

illustrated hence demonstrating its role as an emerging agent of pneumonia. Indeed, a previous study supports the notion that *P. acanthamoebae* is an emerging pathogen as it fulfilled the third and fourth Koch postulates (Casson *et al.*, 2008). The current application of IHC in detecting *P. acanthamoebae* appears to be identifying the etiologic agent for bovine abortions (Blumer *et al.*, 2011; Borel *et al.*, 2007; Lohr *et al.*, 2015; Ruhl *et al.*, 2009; Wheelhouse *et al.*, 2012). Despite the difference in the organism being evaluated, it is proposed that the methods' efficacy would be retained in human samples as well if the pathogen being detected is similar. The emphasis appears to be on employing mice to produce the required antibodies to detect *P. acanthamoebae* and there is no difference in the usage rates of polyclonal and monoclonal antibodies (Blumer *et al.*, 2011; Borel *et al.*, 2007; Casson *et al.*, 2007).

A significant portion of studies done pertaining the use of IHC to detect *P. acanthamoebae* in samples has employed the antibody production protocols which have been developed by Casson and coworkers (Regenscheit *et al.*, 2012; Ruhl *et al.*, 2009; Wheelhouse *et al.*, 2012). The antibody developed by Casson and collaborators is indeed effective when employed to differentiate between distantly related *Chlamydia* like organisms as little or no cross-reactivity was observed (Casson *et al.*, 2007). However, significant cross reactivity was illustrated when the antibodies were utilized to detect *Protochlamydia amoebophila* (*P. amoebophila*) and *P. acanthamoebae* as the two species are rather closely related (Casson *et al.*, 2007). This puts the specificity and sensitivity of the antibody itself under scrutiny as it may contribute to erroneous results in a clinical setting. In the light of this finding, it is possible that other works may have the issue as well as there is an absence of cross-reactivity evaluation. The majority of prior work illustrates that IHC is indeed a valuable tool in the armamentarium against *P. acanthamoebae* hence explaining its widespread use. However, caution should be practiced in interpreting results from IHC due to the possibility of cross-reaction. It is proposed that the employment of this protocol should be followed up with additional testing via molecular techniques such as PCR or FISH.

Table 1. Primers and probes for the detection of *P. acanthamoebae* present in literature.

Technique	Target gene	Primers	Reference
PCR	16S rRNA-encoding gene	PacF, 5'-CTCAACTCCAGAACAGCATTT-3'	(Casson, Posfay-Barbe, <i>et al.</i> , 2008)
		PacR, 5'-CTCAGCGTCAGGAATAAGC-3'	
		PacS, 5'-T [†] TCCACATGTAGCGGTGAAATGCGTAGATAATG-3'	
		PacI, 5'-GAGGTGAAGCAAATCCCCAAA-3'	(Fukumoto <i>et al.</i> , 2010)
		Pac2, 5'-CTCCT [†] TGCGGT [†] TAAGTCAGC-3'	
		FOR2, 5'-CGTGGATGAGGCATGCAAGTCGA-3'	(Wheelhouse <i>et al.</i> , 2011)
		REV2, 5'-CAATCTCTCAATCCGCCTAGACGTCT [†] TAG-3'	
		16SIGF, 5'-CGGCGTGGATGAGGCAT-3'	(Everett <i>et al.</i> , 1999)
		16SIGR, 5'-TCAGTCCCAGTGT [†] TGGC-3'	
		23SAPF2, 5'-GAACCTGAAACCAAGTAGC-3'	(Soldati <i>et al.</i> , 2004)
FISH	23S rRNA-encoding gene	23SAPR, 5'-CTGGCTCATCATGCAAAAGG-3'	
		Adp81F, 5'-TAGTGATCTGCTACGGGAT [†] TT-3'	(Greub, Berger, <i>et al.</i> , 2003)
	ATP/ADP-translocase-encoding gene (tlc)	Adp84R, 5'-TTGGATTAGGATATTGCAAT [†] TT-3'	
		6-FAM-5'-AACCTTGTAGAAGTAACCTGGAAGAACCAGC-3'-TAMRA	
	16S rRNA-encoding gene	Bn ₉ 658, 5'-TCCGTT [†] TCTCCGCCTAC-3'	(Hayashi <i>et al.</i> , 2010; Matsuo <i>et al.</i> , 2008; Sixt <i>et al.</i> , 2012)
		UV7-763, 5'-TGCTCCCCCTTGCT [†] TTCG-3'	
	18S rRNA-encoding gene	EUK516, 5'-ACCAGACTTGCCCTCC-3'	(Hayashi <i>et al.</i> , 2010; Matsuo <i>et al.</i> , 2008; Poppert <i>et al.</i> , 2002)
ISH	16S rRNA-encoding gene	Bn ₉ 658, 5'-TCCGTT [†] TCTCCGCCTAC-3'	(Amann <i>et al.</i> , 1997)

6-FAM is 6-carboxyfluorescein, TAMRA is 6-carboxytetramethylrhodamine.

4. Serology

Serology can be defined as the study of proteins, chiefly antibodies present in blood and secretions such as saliva (Wine *et al.*, 2015). It is minimally invasive and requires the immune system to

operate effectively (Doneley and Health, 2006). Consequently, it only yields results when the presence of a pathogen is significant enough to contribute towards seroconversion (Doneley and Health, 2006). Additionally, serology permits the retrospective confirmation of an infection as it

demonstrates differences between samples from the acute and convalescent phases effectively besides having the capability to identify chronic infections (Maggi *et al.*, 2014; Nilsson *et al.*, 2005). There appears to be an emphasis on employing immunofluorescence for the detection of *P. acanthamoebae* with direct and indirect immunofluorescence having rather similar usage rates (Greub, Boyadjiev, *et al.*, 2003; Hayashi *et al.*, 2012; Marrie *et al.*, 2001; Pilloux *et al.*, 2015). Similar to IHC, immunofluorescence allows visualization of *P. acanthamoebae* in cells or tissues which permits it to be ascribed as an etiological agent for the said disease. Despite the efficacy, care should be taken when interpreting positive results obtained via this technique as cross-reactivity has been reported with *P. amoebaphila* (Casson *et al.*, 2007). As has been previously reported in literature, it is recommended that procedures such as immunoblotting or Western blotting should be employed to ensure truly positive results (Casson *et al.*, 2007; Greub *et al.*, 2009; Greub, Boyadjiev, *et al.*, 2003).

Enzyme-linked Immunosorbent Assay (ELISA) is widely employed as an analytical biochemistry assay. ELISA is rather favored among diagnostic laboratories due to its simplicity, specificity, sensitivity and high throughput nature (Sakamoto *et al.*, 2018). In the context of *P. acanthamoebae*, the approach of employing ELISA for detection has not been well established. Indeed, the closest format of an ELISA identified in literature was that of a multiplexed serology approach which utilized fluorescent probes (Bousbia *et al.*, 2013). Baud and collaborators compared five commercial ELISA kits and identified that one of it which employed 60-kDa chlamydial heat shock protein (cHSP60) as the antigen had cross reactivity with *P. acanthamoebae* (Baud *et al.*, 2010). This suggests the presence of the said protein in *P. acanthamoebae* too albeit probably having different sequences and consequently this opens an avenue for the production of new recombinant antigens specifically for the detection of this intracellular pathogen. Greub *et al.* pioneered work pertaining ELISA for *P. acanthamoebae* when they

pyrosequenced the genome of the bacteria and conducted 2D-gel electrophoresis to identify immunogenic proteins (Greub *et al.*, 2009). They identified a total of 18 proteins in which parachlamydial protein E and N were recombinantly expressed in *E. coli*. The purified recombinant proteins were utilized in a direct ELISA assay and detection was possible with rabbit sera until a dilution value of 1/256. Previous studies have shown that the proteins exhibiting highest antigenicity for *P. acanthamoebae* weigh approximately 48kDa, 60kDa and 70kDa (Casson *et al.*, 2007; Corsaro and Greub, 2006). Information garnered from work by Greub and coworkers can be used to postulate that the proteins weighing 48kDa and 70kDa are elongation factor Tu and chaperonin GroEL respectively (Greub *et al.*, 2009). These proteins have been previously described as being highly immunogenic in *Chlamydia trachomatis* (*C. trachomatis*) (Sanchez-Campillo *et al.*, 1999). Work done on the proteomic aspect of *P. acanthamoebae* infection also revealed elongation factor Tu and Na (+)-translocating NADH-ubiquinone oxidoreductase subunit D as immunogenic proteins (Leitsch *et al.*, 2010). The former is well established as an immunogen while the latter has been identified as an indicator of bacterial virulence in *Chlamydia pneumoniae* (*C. pneumoniae*) and *C. trachomatis* (Häse *et al.*, 2001).

Despite the efficacy of serological techniques in detecting pathogens, there are numerous pitfalls to techniques that fall under this group. The technique is merely a reflection of a patients' immune response and hence cannot provide an information on the microbial load or severity of infection (Fierz, 2004). It may also have questionable use in clinical settings as the immune response of an individual is commonly polyclonal in nature hence potentially rendering antigens present in a test panel ineffective (Fierz, 2004). However, as serology based diagnosis of *P. acanthamoebae* is already present, it is suggested that better suited antigen targets should be sought after to potentially improve the usage rates of these techniques in detecting *P. acanthamoebae*.

Table 2. An overview of the advantages and limitations of various techniques employed in the isolation of *P. acanthamoebae*.

Technique	Advantages	Limitations	References
Culture-based isolation	<ul style="list-style-type: none"> Permits recovery of actual strains of the pathogen. Existence of viable organisms can be illustrated. Population size can be inferred from the sample. Influence of the pathogen on cells can be assessed if cell culture is utilized. High sensitivity if appropriate isolation panel is used. 	<ul style="list-style-type: none"> Technique and operator sensitive. Risk of contamination leading to erroneous results. Time consuming as there is an incubation period prior to illustration of the organisms' presence. Efficacy is limited in detecting fastidious organisms. 	(Figdor and Gulabivala, 2008; Kaur and Kaur, 2015)
PCR	<ul style="list-style-type: none"> Permits detection of pathogens that would be culture-negative. Allows intra-species discrimination. High sensitivity. High specificity. Lower risk of contamination Usage of qPCR would permit accurate quantification of microbial load in samples. 	<ul style="list-style-type: none"> Possibility of false negatives and false positives. Unable to discriminate between live and dead organisms. Prone to human error. 	(Berridge <i>et al.</i> , 1998; Hoffmann <i>et al.</i> , 2010; Kralik and Ricchi, 2017; Madico and Rice, 2008; Maneg <i>et al.</i> , 2016; Morillo <i>et al.</i> , 2003; Navarro <i>et al.</i> , 2004; Riffard <i>et al.</i> , 1998; Valasek and Repa, 2005; Valones <i>et al.</i> , 2009)
FISH	<ul style="list-style-type: none"> Possible to detect and identify pathogen in clinical samples without any form of cultivation. Permits identification of pathogen in clinical cases with polymicrobial infections. Technical requirement for the assay is low and hence permits usage in resource-limited areas. Can be developed to detect antimicrobial-drug resistance that are due to rRNA gene mutations. Useful in detecting fastidious organisms. Cost-effective in comparison with PCR. 	<ul style="list-style-type: none"> Requirement for an experienced and trained individual. Sensitivity is considerably below that of PCR in the analysis of primary materials. Increased difficulty in using it to pinpoint non-ribosomal antimicrobial-drug resistance mechanisms due to considerably lower concentration of such mRNAs in comparison with rRNAs. 	(Frickmann <i>et al.</i> , 2013, 2017; Fritsche <i>et al.</i> , 1999; Horn <i>et al.</i> , 1999; Juretschko <i>et al.</i> , 1998; Poppert <i>et al.</i> , 2002; Snaidr <i>et al.</i> , 1997; Yilmaz and Demiray, 2007)

IHC	<ul style="list-style-type: none"> • The role of an organism in an illness can be illustrated vividly. • Can be used on patients awaiting culture results. • Possible to be used on samples with low bacterial loads. • Can be utilized on patients that have already been partially treated. • A permanent record of the sample can be stored. 	<ul style="list-style-type: none"> • Possibility of cross-reaction. • Results can be ambiguous due to nonspecific or weak staining. 	(Casson <i>et al.</i> , 2007; Ellis <i>et al.</i> , 2005; Guarner <i>et al.</i> , 2000; Kundu <i>et al.</i> , 2014)
Serology	<ul style="list-style-type: none"> • Minimally invasive. • Possible to identify chronic infections. • Discriminates between samples from acute and convalescent phases. • The role of an organism in an illness can be illustrated vividly when immunofluorescence is used. • Only yields response when a pathogens' presence is significant enough to cause seroconversion hence minimizing false positives. 	<ul style="list-style-type: none"> • Possibility of cross-reaction. • Probability of false positives and false negatives. • The validity of a serology based test can only be established for a defined population. • The antigen used as the target highly affects the tests' performance. 	(Doneley and Health, 2006; Fierz, 2004; Maggi <i>et al.</i> , 2014; Nilsson <i>et al.</i> , 2005)

CONCLUSION

Over the years, the number of cases of CAP has experienced a rather significant increase and adding to the issue would be the various etiologic agents that cause the ailment. The present findings illustrate the multiple techniques present for the detection of *P. acanthamoebae*, in both human and animal samples. Succinctly, each method has its own sets of pros and cons. Therefore, it is proposed that detection procedures should be employed based on the clinical presentation of the patient and response to empirical therapy as the bacteria is an atypical agent of pneumonia. It is suggested that comparative analysis pertaining the efficacy of different detection techniques should be conducted to identify the situations in which they would be effective in. Additionally, further research should be done to develop novel detection techniques for the said pathogen as it would contribute towards improved detection of intracellular pathogens as a whole.

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