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Multiplex PCR: Improving Microbiological Diagnosis, A Review

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Abstract

nucleic acid segments present in small or complex samples to sufficiently high levels to allow their detection by optical, physical and other methods. It is a rapid and simple way of copying specific DNA fragments from minute quantities of source DNA material using a heat-stable polymerase and suitable primers to direct the amplification of the desired region of DNA. **Objective:** The objective is to review multiplex PCR and its application in diagnosis of disease. **Method:** The method adopted in this review is broad search of both electronic data bases, hard copies by studying and comparing the information obtained from available current published scientific researchers in peer reviewed journals, books, and conferences etc. Results: it was found that Diagnostic microbiology is in the midst of a new era. Rapid nucleic acid amplification and detection technologies are quickly displacing the traditional assays based on pathogen phenotype rather than genotype. **Conclusion:** The review concludes that PCR technology is gradually changing the world of rapid laboratory diagnosis.

Keywords
Multiplex, PCR, Diagnosis, Microbiological

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INTRODUCTION

The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence (Bartlett and Stirling, 2003). PCR can be performed using source DNA from a variety of tissues and organisms, including peripheral blood, skin, hair, saliva, and microbes. Only trace amounts of DNA are needed for PCR to generate enough copies to be analyzed using conventional laboratory methods. For this reason, PCR is a sensitive assay (Garibyan and Avashia, 2013).

Steps in PCR

There are three major steps involved in the PCR technique: denaturation, annealing, and extension. In step one; the DNA is denatured at high temperatures (from 90 - 97 degrees Celsius). In step two, primers anneal to the DNA template strands to prime extension. In step three, extension occurs at the end of the annealed primers to create a complimentary copy strand of DNA. This effectively doubles the DNA quantity through the third steps in the PCR cycle. To amplify a segment of DNA using PCR, the sample is first heated, so the DNA denatures, or separates into two pieces ofsingle-stranded DNA. Next, an enzyme called "Taq polymerase" synthesizes - builds - two new strands

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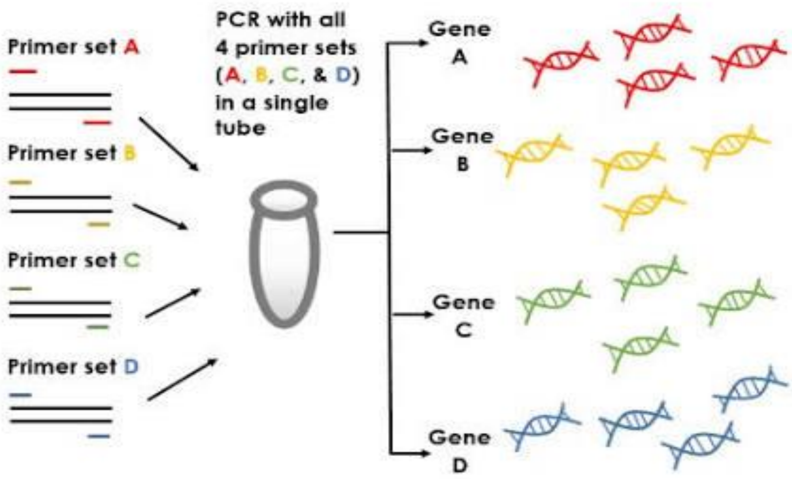
of DNA, using the original strands as templates. This process results in the duplication of the original DNA, with each of the new molecules containing one old and one new strand of DNA. Then each of these strands can be used to create two new copies, and so on, and so on (Joshi and Despande, 2010).

In diagnostic laboratories the use of PCR is limited by cost and sometimes the availability of adequate test sample volume. Toovercome these shortcomings and also to increase the diagnostic capacity of PCR, a variant termed multiplex PCR has beendescribed (Elnifro *et al*., 2000).

Multiplex PCR

Multiplex PCR systems are increasingly used in biological and medical studies as they allow simultaneous amplification of several DNA fragments within one reaction. This ability to reduce the number of reactions needed to test a sample for different targets helps saving time and money and makes multiplex systems useful especially when large sample numbers have to be screened (Sint *et al*., 2012).



Overview of multiplex PCR (MacDonald and Jerris, 2018).

Types of multiplex PCR

1. Single template multiplex PCR: This technique uses a single template, which can be a genomic DNA, along with several pairs of forward and reverse primers to amplify specific region within the template.
2. Multi-template Multiplex PCR: This technique uses multiple templates and several pairs of forward and reverse primers for each template and regions within the template, in the same reaction tube (Bagwe and Nagendra, 2017).

Applications of multiplex PCR technique

1. Examining population genetics and parentage assignment (Guichoux *et al*., 2011)
2. Investigate trophic interactions (Macfadyen *et al*., 2009),
3. For molecular species identification (Staudacher *et al*., 2011)
4. Community assessment (Gioia *et al*., 2010)
5. Forensics (Hill *et al*., 2009)
6. Food safety studies (Randhawa *et al*., 2009).

The considerable potential of this method is also reflected in the rapidly rising numbers of publications that have adopted this approach.

Multiplex PCR in diagnosis of bacterial infections

We believe that multiplex nucleic acid amplification to detect a panel of putatively pathogen traits should be considered as a replacement for tedious, less sensitive, and less specific detection technologies in clinical and food microbiologic analyses (Catalina *et al*., 2003).

1. Bacterial Vaginosis

   Women with bacterial vaginosis are at increased risk for adverse pregnancy outcomes and genital infections such as chorioamnionitis, premature rupture of membranes, premature delivery, miscarriage in the first trimester after in vitro fertilization, and post-operative upper genital tract infections. Furthermore, bacterial vaginosis is associated with an increased risk for human immunodeficiency virus (HIV)-1 infection and urinary tract infections (Mana *et al*., 2002).

   We have developed a multiplex PCR–based diagnostic test for bacterial vaginosis involving the detection of multiple anaerobes in vaginal swab samples. The test design is based on the fact that *Bacteroides fragilis*, and *Gardnerella vaginalis* are the most common anaerobes associated with bacterial vaginosis, and that their predominance in the vaginal flora is indicative of bacterial vaginosis (Mana *et al*., 2002).

2. Diarrhoeagenic Bacteria

There is a wide range of recognized enteric pathogens such as viruses, bacteria, and parasites that cause diarrhea. Among bacteria, Shigella spp. And diarrheagenic Escherichia coli (DEC) are the most common causes of diarrheal diseases in developing countries (Davidson et al., 2002). The human diarrheagenic *Escherichia coli* strains are classified into six major categories which include shiga toxin producing E. coli (STEC), enterotoxigenic E. coli (ETEC), enteropathogenic E. coli (EPEC), enteroinvasive E. coli (EIEC), enteroaggregative E. coli (EAggEC) and diffusely adherent E. coli (DAEC) (Smith and Fratamico, 2005).

A multiplex polymerase chain reaction (mPCR) was developed for identification of DEC infections caused by shiga toxin producing E. coli (STEC), enteropathogenic E. coli (EPEC), enterotoxigenic E. coli (ETEC), enteroinvasive E. coli (EIEC) and Shigella in feacal samples by simultaneous and specific detection of seven virulence genes (eaeA, stx1, stx2, bfpA, LT, ST and ipaH) (Helmy *et al*., 2013).

3. Bacterial pathogens of the urinary tract

The most frequently isolated bacteria responsible for urinary tract infection (UTI) are coliform organisms – about 70% of all bacterial strains isolated from urine samples, followed by Enterococcus spp. – about 15.0%, coagulase-negative staphylococci - about 10% and *Pseudomonas aeruginosa*- about 5%. Among the coliforms, *E. coli* comprise about 75% of isolates (Cybulski and Kjaeldgaard, 2010).

The urine culture is still the "gold standard" for diagnosis of UTI (Bartges, 2004). It is simple and inexpensive. Moreover, many bacteria which are responsible for UTI can easily grow on the medium. However, sensitivity, quality of the medium, risk of interpretation errors by culture and time-consuming growing are limitations. The cultures require 24 to 48 hours to provide results after pure cultures are obtained (Lehmann, 2010).

The development of molecular techniques has considerably improved the rapidity and accuracy of the microbiological diagnostics. PCR is simple, highly specific, sensitive and amenable to full automation. It has been successfully used to detect bacterial DNA from different biological fluids. Compared with the classical urine culture, PCR is more rapid and the results are available 5 hours after the specimen collection. However, the use of PCR in diagnostic laboratories is limited by cost, availability of adequate diagnostic kits and availability of appropriate biological materials (Elnifro *et al*., 2000).

The multiplex PCR method used here proved to be highly specific since it gave only 3.1% of false positive results in comparison with urine cultures of monomicrobial infections and infections caused with two bacterial strains (Cybulski and Kjaeldgaard, 2010).

Multiplex PCR in Viral diagnosis

1. Neurotrophic Viruses:

Viruses are the main etiological cause of central nervous system (CNS) infections, ahead of bacterial and fungal causes (Somand and Meurer, 2009). They are responsible for encephalitis and aseptic meningitis (Steiner, 2011). Encephalitis is a rare but one of the most devastating neurological disorders. Encephalitis in humans is due mainly to herpesviruses, in particular herpes simplex (HSV) and varicella-zoster (VZV) viruses, but also to cytomegalovirus (CMV), Epstein-Barr virus (EBV), or human herpesvirus 6 (HHV-6) in the immunocompromised host (Kleinschmidt-DeMasters and Gilden, 2001). Human enteroviruses (HEVs) are the most common cause of aseptic meningitis outbreaks in children during the summer season (Antona *et al.*, 2007)

Molecular techniques are now considered the gold standard for the detection in CSF samples of the viruses responsible for CNS infections (Kimberlin, 2005). The molecular tests used in routine diagnosis have to be specific and highly sensitive, allowing rapid and valuable detection of RNA and DNA viruses. PCR has proved to be a powerful tool for investigating meningitis and encephalitis caused by a variety of viruses. Inneurological disease the requirement of rapid and reliablediagnosis to provide a rational basis for chemotherapy andlimit unnecessary procedures and irrelevant therapy has drivendevelopment (Elnifro, 2000).

2. Respiratory Viruses:

Acute respiratory tract infections (ARI) are a leading cause of morbidity and mortality in children worldwide accounting for about 30% of all childhood deaths in developing world. Viruses account for 50–90% of acute lower respiratory tract infections (ALRI) in young children with *Respiratory syncytial virus* (RSV), parainfluenza viruses (PIV), influenza viruses A and B and human metapneumoviruses (hMPV) being most commonly identified (Broor and Bharaj, 2007).Respiratory infections caused by above said viruses usually present with clinical features that are nearly indistinguishable (Debbia *et al*., 2001).The increased sensitivity of polymerase chain reaction (PCR) over conventional methods for the diagnosis of respiratory viral infections has been established previously (Weinberg*et al*., 2004). However, organism-specific RT-PCR assays which require separate amplification of each virus under investigation are resource intensive, time consuming and labor intensive (Templeton *et al*, 2004).

The development of multiplex PCR for the detection of respiratory viruses as a rapid, sensitive and time saving technique has not gained priority in India even though ~0.5 million children die each year in this country due to ALRI each year, accounting for one fourth of the 1.9 million global ALRI deaths (Bharaj *et al*., 2009).

3. Genito-Urinary Viral infections

The utility of multiplex PCR in diagnosis of viruses associated with genital tract infection is reflected by the numerous reports detecting and typing human papilloma viruses (HPVs) (Elnifro *et al*., 2000). A multiplex PCR that combined the detection of C. trachomatis and two viruses (HPV and HSV) was developed, optimized, and evaluated using cervical and endocervical specimens from patients suspected to be infected with one or more of these agents. The testproduced 100% correlation with the results of the uniplex PCRs in 92 genital swabs (29 were positive for HSV, 16 were positive for HPVs, and one was positive for C. trachomatis). In addition, a co-infection with HPV and HSV was detected by the multiplex PCR. In other studies primer pairs for HSV were combined with those for *Haemophilus ducreyi* and *Treponema pallidum* to construct a multiplex PCR for diagnosis of genital ulceration (Elnifro *et al*., 2000).

Multiplex PCR in diagnosis of Parasitic infections

1. Entamoeba species

E. histolytica, the pathogenic amoeba, is indistinguishable in its cyst and trophozoite stages from those of non-pathogenic *E. dispar* and *E. moshkovskii*, except in rare cases of invasive disease when *E. histolytica* trophozoite may contain ingested red blood cells. Estimates of intestinal *E. histolytica* infections have been primarily based on microscopic examination of stools, which has a sensitivity of only 60%, even under optimal standards (Krishna and Subhash, 2007).

We described a new PCR-based approach for accurate detection and differentiation of the two species that compose the *E. histolytica/E. dispar* complex, by extracting DNA directly from stool samples without prior cultivation. The protocols are fast and simple, no laborious hybridizations or use of radionucleotides are required, and the entire procedure can be performed in one day (Yury*et al*., 2001).

The nested multiplex PCR developed and evaluated in the present study showed that the size of diagnostic fragments of PCR products was clearly different for all the three Entamoeba species, the species-specific product size for *E. histolytica*, *E. moshkovskii* and *E. dispar* was 439, 553 and 174 bp respectively (Krishna and Subhash, 2007).

2. Plasmodium species

Conventional light microscopy using Giemsa-stained blood films is a simple method and considered as the ‘gold standard’ to detect malaria parasites. This method is still being used to diagnose malaria as it can determine parasite morphologies and differentiate stages among Plasmodium species. However, it is time-consuming, it requires a well-trained microscopist, and the accuracy varies depending on the expertise of the microscopist. Misdiagnoses may occur when there is mixed infection and low levels of parasitemia (Cox, 2010).

Even though the rapid diagnostic test (RDT) performs a fast detection of Plasmodium species, it has limitations in identifying all of the 5 human malaria species, with only *P. falciparum* and *P. vivax* being identified without problems (Moody, 2002).

Polymerase chain reaction (PCR) offers a good alternative to microscopy in terms of sensitivity and specificity and could be used as a confirmatory tool for microscopy identification of malaria parasites (Johnston, 2006).

In this multiplex system the mitochondrial cytochrome b gene for P. falciparum was targeted because it has a highly conserved region. The high degree of conservation might be due to structural constraints on the genome and the fact that their genome size and gene arrangement remain unchanged for a long period of time(Hikosaka *et al*., 2011).Therefore, with improved PCR technique, the cytochrome b gene can also be targeted (Steenkeste *et al*., 2009). The dihydrofolate reductase gene was targeted in identifying *P. knowlesi* (Berry*et al*., 2005).

Multiplex PCR in diagnosis of Fungal infections

1. Candida species

Candida species are prevalent in the oral cavity. *C.albicans* is the most common species isolated from oral cavity in both healthy and those suffering from various respiratory tract disorders. In a special condition Candida becomes an opportunistic human pathogen and cause candidiasis, which colonizes in several histological special sites, including the skin, oral cavity, esophagus, digestive tract, vagina and blood vessels of humans (Mohammed *et al*., 2016).

The use of PCR system with the species- specific primer mixes is rapid and it simplifies the assay further than that of PCR using a single pair of primers, because it is possible to identify unambiguously 10 species of Candida from just three sets of primers (Kambe *et al*., 2002). In this perspective, multiplex PCR can represent a valid solution. The availability of more primers could potentially allow the identification of all the species of Candida with the accuracy of a genetic system and without the costs of other molecular methods (i.e. sequencing), which limit their widespread use (Liguori *et al*., 2010).

Primer design for multiplex PCR

Primer design for multiplex PCR is still a challenging problem and several factors need to be considered. These problems include:

1. Mis-priming due to nonspecific binding to non-target DNA template
2. Primer dimerization
3. Inability to separate and purify DNA amplicons with similar electrophoretic mobility (Shen *et al*., 2010).

Relative decrease in sensitivity is one of the limitations of this method in comparison with single PCR method (Hajia, 2017).

CONCLUSION

Multiplex PCR is gradually changing the world the world of rapid laboratory diagnosis by

1. Reducing turnaround time
2. Reducing labour
3. Saving cost.

Recommendation

The incorporation of PCR in the diagnosis of disease should be introduced to facilitate rapid diagnosis and effective treatments.

REFERENCES

Alanio, A. and Bretagne, S. Performance evaluation of multiplex PCR including Aspergillus—not so simple. Medical Mycology, 55(1): 56-62.

Antona, D., Lévêque, N., Chomel, J.J., Dubrou, S., Lévy-Bruhl. And Lina. B. 2007. Surveillance of enteroviruses in France, 2000–2004. European journal of clinical microbiology and infectious diseases. 26:403–412.

Bagwe, P., Nagendra, P. (2017). Multiplex PCR and its applications. https://www.slideshare.net/nagendragowda/multiplex-pcr-and-its-applications Available at Retrieved on 16th May, 2018.

Bartges, J.W.2004. Diagnosis of urinary tract infections. Veterinary Clinics of North America: Small Animal Practice,34(4): 923-33.

Bartlett, J. M. S., & Stirling, D. 2003. Methods in Molecular Biology. A short history of the polymerase chain reaction, 226, 3-6.

Berry, A., Fabre, R., Benoit-Vical, F., Cassaing, S. and Magnaval, J.F. Contribution of PCR-based methods to diagnosis and management of imported malaria. Medecine Tropicale 2005; 65(2): 176– 183.

Bharaj, P., Sullender, W.M, Kabra, S.K., Mani, K., Cherian, J., Tyagi, V., Harendra, S.C., Kaushik, S., Dar, L. and Broor, Shobha. (2009). Respiratory viral infections detected by multiplex PCR among pediatric patients with lower respiratory tract infections seen at an urban hospital in Delhi from 2005 to 2007. Virology Journal, 6:89.

Broor, S. and Bharaj, P. (2007) Avian and human metapneumovirus. Annals of the NewYork academy of sciences, 1102:66-85.

Catalina, L., Jorge, F.C., Nicolas, V., Rocío, T., Raul, V., Javier, T., Phillip I. T. and Teresa, E. (2003). Single Multiplex Polymerase Chain Reaction To Detect Diverse Loci Associated with Diarrheagenic Escherichia coli. Emerging Infectious Diseases.9(1), 127-131.

Cox FEG. 2010. History of the discovery of the malaria parasites and their vectors. Parasite Vectors.3(1): 5.

Cybulsk5i, Z. and Kjaeldgaard, P. 2010. Resistance to oral antibiotics in urinary coliform strains isolated between 2000 and 2008 in Sønderborg area, Denmark. International Journal of Antimicrobial Agents,35(5): 516-7.

Davidson, G., Barnes, G., Bass, D., Cohen, M., Fasano, A., Fontaine, O. and Guandalini, S. (2002). Infectious diarrhea in children: working group report of the First World Congress of Pediatric Gastroenterology, Hepatology, and Nutrition. J. Pediatr. Gastroenterol. Nutr. 35(Suppl. 2):S143-S150.

Debbia EA, Schito GC, Zoratti A, Gualco L, Tonoli E, Marchese A. (2001). Epidemiology of major respiratory pathogens. Journal of Chemotherapy, 1(1):205-10.

Elnifro, M.E., Ashshi, A.M., Cooper, R.J. and Paul, E.K. (2000). Multiplex PCR: Optimization and application in diagnostic virology. Clinical Microbiology Reviews, 13(4): 559-570.

Garibyan, L. and Avashia, N. (2013). Research techniques made simple: Polymerase chain research (PCR). Journal of investigation in dermatology, 133(3): e6. doi:10.1038/jid.2013.1

Gioia, G., Lecova, L., Genchi, M., Ferri, E., Genchi, C. and Mortarino, M. (2010) Highly sensitive multiplex PCR for simultaneous detection and discrimination of Dirofilaria immitis and Dirofilaria repens in canine peripheral blood. Veterinary Parasitology, 172, 160–163.

Guichoux, E., Lagache, L., Wagner, S., Chaumeil, P., Leger, P., Lepais, O., Lepoittevin, C., Malausa, T., Revardel, E., Salin, F. and Petit, R.J. (2011). Current trends in microsatellite genotyping. Molecular Ecology Resources, 11, 591–611.

Hajia, M. (2017). Limitations of different PCR protocols used in diagnostic laboratories: A short review. Modern Medical Laboratory Journal, 1(1): 1-6.

Helmy, O.M., Ragab, Y.M. and Hussein, M.M.M. 2013. Multiplex polymerase chain reaction (PCR) for the detection of diarrheagenic Escherichia coli and Shigella directly from stool. Afr. J. Microbiol. Res, 7(34): 4368-4372.

Hikosaka K, Watanabe YI, Kobayashi F, Waki S, Kita K, Tanabe K. 2011. Highly conserved gene arrangement of the mitochondrial genomes of 23 Plasmodium species. Parasitology International, 60(2): 175– 180.

Hill, C.R., Butler, J.M. and Vallone, P.M. (2009) A 26plex autosomal STR assay to aid human identity testing. Journal of Forensic Sciences, 54, 1008–1015.

Hiroko, T., Yuriko, Y., Miho, N., Yasuhiro, T., Toshiharu, S., Souichi,D., Norio, S.and Takayuki T. 2016. Multiplex Polymerase Chain Reaction Assay for Early Diagnosis of Viral Infection. Available at http://dx.doi.org/10.5772/65771. Retrieved on 21st May 2018.

Jaskula, E., Dlubek, D., Sedzimirska, M., Duda, D., Tamouska, A. and Lange, A. 2010. Reactivations of cytomegalovirus, human herpesvirus 6, and Epstein-Barr virus difer with respect to risk factors and clinical outcome after hematopoietic stem cell transplantation. Transplantation Proceedings, 42:3273-3276.

Johnston SP, Pieniazek NJ, Xayavong MV, Slemenda SV, Wilkins PP, Silva AJ. 2006. PCR as a confirmatory technique for laboratory diagnosis of malaria. Journal ofClinical Microbiology, 44(3): 1087–1089.

Joshi, M. and Despande, J.D. (2010). Polymerase chain reaction: methods, principles and applications. International Journal of biomedical research, 1(5): 81-97.

Kanbe T, Horii T, Arishima T, Ozeki M, Kikuchi A.2002. PCR-based identification of pathogenic Candida species using primer mixes specific to Candida DNA topoisomerase II genes. Yeast, 19(11): 973- 89.

Kimberlin, D. W. (2005). Diagnosis of herpes simplex virus infections of the CNS. Expert Rev. Mol. Diagn. 5:537–547.

Kleinschmidt-DeMasters, B. K., and D. H. Gilden. 2001. The expandingspectrum of herpesvirus infections of the nervous system. Brain Pathology.11:440–451.

Krishna, K. and Subhash, C.P. 2007. A novel nested multiplex polymerase chain reaction (PCR) assay fordifferential detection of Entamoeba histolytica, E. moshkovskii and E. dispar DNA in stool samples. Biomedical Central microbiology, 7(47). doi: 10.1186/1471-2180-7-47.

Lehmann, L.E., Hauser, S., Malinka, T., Klaschik, S., Stüber, F and Book, M.2010. Real-time polymerase chain- reaction detection of pathogens is feasible to supplement the diagnostic sequence for urinary tract infections. British Journal of UrologyInternational, 106(1): 114-20.

Liguori, G, Gallé F, Lucariello, A, Valeria, D.O, Albano L, Mazzarella G, D'Amora, M. and Rossano, F. Comparison between multiplex PCR and phenotypic systems for Candida spp. Identification. New Microbiologica, 33(1): 63-67.

MacDonald, H. and Jerris, R.C. 2018. PCR Fundamentals: Focus on Multiplex PCR Assay and the Advantages over Singleplex Assays. Media Lab Incorporated. Available at http://medialabinc.net/pcr_fundamentals.aspx. Retrieved on 24th May, 2018.

Macfadyen, S., Gibson, R., Raso, L., Sint, D., Traugott, M. and Memmott, J. (2009) Parasitoid control of aphids in organic and conventional farming systems. Agriculture, Ecosystems and Environment, 133, 14–18.

Mana, O., William, B., Hiromi, H. and Hideo, H. 2002. A multiplex polymerase chain reaction-based diagnostic method for bacterial vaginosis. Obstetrics and Gynecology, 100(4): 759-764.

Mohammed, A.B., Ali, J.H. and Abdullah, S.K. 2016. Multiplex polymerase chain reaction identification of Candida species colonized sputum of patients suffering from various respiratory tract disorders in Duhok, Iraq. International Journal of Research in Medical Sciences, 4(5): 1558-1563.

Moody A. 2002. Rapid diagnostic tests for malaria parasites. Clinical Microbiology Reviews, 15(1): 66–78.

Randhawa, G.J., Chhabra, R. and Singh, M. (2009) Multiplex PCR-based simultaneous amplification of selectable marker and reporter genes for the screening of genetically modified crops. Journal of Agricultural and Food Chemistry, 57, 5167–5172.

Sharma, N. K., C. E. D. Rees, and E. R. Dodd. 2000. Development of a single-reaction multiplex PCR toxin typing assay for Staphylococcus aureus strains. Applied and environmental microbiology. 66:1347–1353.

Shen, Z., Qu, W., Wang, W., Lu, Y., Wu, Y., Li, Z., Hang, X., Wang, X., Zhao, D. and Zhang, C. (2010). MP primer: A program for reliable multiplex PCR primeer design. BMC Bioinformatics, 11: 143.

Sint, D., Raso, L. and Traugott, M. (2012). Advances in multiplex PCR: balancing primer efficiencies and improving detection success. Methods in Ecology and Evolution, 3: 898-905.

Smith, J.E., Fratamico, P.M. (2005). Diarrhea-inducing Escherichia coli. In Foodborne pathogens: microbiology and molecular biology. Caister Academic Press, Norfolk, UK. pp. 357-382.

Somand, D., and W. Meurer. 2009. Central nervous system infections. Emergency medicine clinics of North America. 27:89–100.

Staudacher, K., Pitterl, P., Furlan, L., Cate, P.C. and Traugott, M. (2011) PCR-based species identification of Agriotes larvae. Bulletin of Entomological Research, 101, 201–210.

Steenkeste, N., Incardona, S., Chy, S., Duval, L., Ekala, M.T., Lim, P., Hewitt, S., Sochantha, T., Socheat, D., Rogier, C., Puijalon, O.M., Fandeur, T. and Ariey, F. 2009. Towards high-throughput molecular detection of Plasmodium: new approaches and molecular markers. Malaria Journal,8: 86.

Steiner, I. 2011. Herpes simplex virus encephalitis: new infection or reactivation? Current opinion in neurology.24:268–274.

Templeton, K.E, Scheltinga, S.A, Beersma, M.F, Kroes, A.C, Claas, E.C. (2004) Rapid and sensitive method using multiplex real-time PCRfor diagnosis of infections by influenza A and influenza B viruses, respiratory syncytial virus, and parainfluenza viruses 1, 2, 3, and 4. Journal of Clinical Microbiology, 42(4):1564-9.

Weinberg, G.A, Erdman, D.D, Edwards,K.M, Hall, C.B, Walker, F.J, Griffin, M.R. and Schwartz, B. (2004). New Vaccine Surveillance Network Study Group: Superiority of reverse-transcription polymerase chain reaction to conventional viral culture in the diagnosis of acute respiratory tract infections in children. Journal of Infectious Diseases, 189(4):706-10.

Yury O. N., Maria, A.F., Diamela T., Jose, A. S., Ivon, M., Jorge, L.M. and Luis, F. Multiplex Polymerase Chain Reaction Amplification And Differentiation Of Entamoeba Histolytica And Entamoeba Dispar Dna From Stool Samples. American journal of tropical medicine and hygiene, 64(5): 293-297.