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Basics of molecular biology and its applications: III. Polymerase chain reaction and in situ hybridization

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Immunohistochemistry (IHC) has been successfully used to detect protein products and infective, microbial or viral particles in cells. One of its limitations is its inability to detect these agents when they are present in minute quantities. In situ hybridization (ISH) has an advantage over immunohistochemistry in its ability to demonstrate specific messenger RNA (mRNA). ISH can be combined with IHC in the same section to detect gene products as well as the mRNA encoding them. The polymerase chain reaction (PCR) offers an even more sensitive method. Genes code for proteins which are the final synthetic products expressed in the cells. Increased expression of the genes is reflected by an increased synthesis of protein products and higher levels of expression of transcribed mRNA. This principle is utilized in the application of ISH and PCR for the diagnosis of disease. The techniques of ISH and PCR are potentially useful in any situation that requires the examination of RNA or DNA. They have been used in the study of infectious, malignant and genetic diseases. With recent modifications and refinements, these techniques have transcended from the level of research tools to use in routine diagnostic laboratories.

Both these techniques have already been extensively used in the field of diagnostic laboratory work. They continue to play a vital role not only in the diagnosis but also in the understanding of the molecular basis of disease. In this article both techniques are described and their applications are considered.

In situ hybridization

The in situ hybridization procedure was first described 24 years ago at the end of a decade in which most of the now commonly used hybridization methods were first reported. The technique's potential was quickly realized and within a few years it had been used for the identification of genomic, viral and mRNA sequences in cryostat, paraffin wax and electron microscopic preparations.

In situ hybridization is a procedure in which specific nucleic acid sequences can be identified within a tissue. It follows the same basic principle of reassociation of probe and target single-stranded DNAs. The nucleic acid probes available are of the isotopic type such as ^{32}P, ^{35}S, ^{3}H and ^{125}I or nonisotopic ones such as biotin, fluorescein or digoxigenin. After the hybridization of target and the probe, results can be detected through autoradiography (for the isotopic ones) or enzymatic reactions. In nonisotopic probes, the method employs capillary action technology and one can identify the exact cellular location of a specific nucleic acid sequence in a tissue or cells.

Oligonucleotides are short segments of DNA or RNA with a known specific sequence that can be chemically synthesized. As with all nucleic acids, this sequence has a 5' end and a 3' end. The sequence can be labelled with a reporter group and localized in a hybridization reaction.

Oligonucleotide probes are commonly employed as they have several advantages in a clinical laboratory setting. They are cost-effective, single-stranded, easy to handle and site specific. The probe can be synthesized on an automated DNA synthesizer. Nonisotopic biotin-labelled oligonucleotide probes are

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preferred to the isotopic ones as the assay can be done within a few hours compared to 4-30 days for radiolabelled ones. Moreover, the probe can be stored for a year and disposal of the radioactive material is not needed.

The Code-on automated molecular pathology system is available for in situ hybridization but is an expensive piece of equipment. The cost of a manual microprobe system is relatively moderate. The basic pieces of equipment required are an incubator of 37-100°C for proteolytic digestion, denaturation and hybridization of DNA, a heating block, a fume hood for handling toxic solutions and an autoclave for preparation of nuclease-free solutions. Although expensive reagents are used, they are consumed in such small volumes that on a case-to-case basis the costs are minimal. The automated system allows further reduction in the overall cost if a large number of cases are performed at the same time.

In the past, frozen sections were employed for hybridization but the morphology of the tissues is not well preserved and in such a procedure the results are also difficult to interpret. Hence, nowadays aldehyde-based fixatives such as formalin, Bouin's fluid or glutaraldehyde are more commonly used. The tissues are fixed, processed, embedded in paraffin, cut at 5 microns and placed on slides. They are air dried. The paraffin is removed by dewaxing and the tissue rehydrated. To break up protein-nucleic acid complexes the tissue is treated with a low concentration of hydrochloric acid and protease. Denaturation of the DNA is achieved by heating the slides to 100°C. The probe is diluted in a formamide-free probe diluent and hybridized for 60 minutes at 55°C in a moist chamber. This is followed by post-hybridization washing to remove the nonhybridized probe and nonspecific hybridization products. For biotin-labelled probes, signal detection is achieved by streptavidin conjugated to either alkaline phosphatase or horseradish peroxidase. By means of chromogen systems which change colour with the enzymes, detectable signals are generated. The slides are then counterstained. Simultaneous controls are used to insure probe specificity.

The precautions to be taken during the ISH procedure, even an automated one, are: (i) the probes should be tried at full strength in some cases; (ii) the hybridization time should be increased in a few selected cases for a better result; (iii) the minimum denaturation time should be at least 8 minutes and this can be increased to 10-12 minutes; (iv) before counterstaining, slides should be checked to find out whether the chromogen reaction has gone to completion; and (v) the tissues should be covered with the probe solution throughout the hybridization procedure, otherwise the tissues will dry out and the reaction will not occur.

The successful use of ISH was first reported by Brigati et al. (1). They used biotinylated DNA probes for the detection of herpes simplex virus and adenoviruses in confirming the diagnosis of viral pneumonia in postmortem lung sections. Myerson et al. (2) demonstrated cytomegalovirus (CMV) in two patients who underwent bone marrow transplantation for leukaemia. Cytological smears of bronchial lavage specimens have also been used for the diagnosis of CMV by ISH. The subtyping of human papillomavirus genotypes by ISH has made a significant contribution to our knowledge of cervical intraepithelial neoplasia (CIN) and carcinoma of the cervix. The human papillomavirus (HPV) genotypes 16, 18 and 31 have been demonstrated in high-grade CIN (CIN III) and in in situ and invasive squamous cell carcinoma of the cervix. This has provided the guidelines for follow-up protocols of patients with CIN lesions. Cytological specimens and cervical smears have been used for this purpose. The aetiological relationship of HPV with carcinoma of the cervix has been more clearly elucidated with the demonstration of these genotypes in CIN III and carcinoma. The HPV genotypes 6 and 11 have been demonstrated in low-grade CIN lesions and genital condylomata. Formalin-fixed paraffin-embedded sections of genital condylomata yielded more positivity for HPV DNA by ISH than by IHC, confirming the sensitivity of this technique (3). HPV genotypes have also been demonstrated in anogenital carcinoma (HPV 16, 18), juvenile laryngeal papillomatosis (HPV 6, 11), carcinoma of the larynx (HPV 16, 18, 31) and in some other neoplastic lesions. The Epstein-Barr virus (EBV) has been identified in Burkitt's lymphoma by this technique. In Papua New Guinea HPV

genotypes have been studied in relation to juvenile laryngeal papillomatosis and carcinoma of the cervix (4).

ISH has been used in pathological specimens from AIDS (acquired immune deficiency syndrome) patients to detect CMV DNA in kidney sections and in a small number of Kaposi's sarcoma lesions. Nakamura et al. (5) demonstrated JC virus DNA in brain sections of progressive multifocal leukoencephalopathy by radiolabelled and biotinylated probes. Hepatitis B virus (HBV) DNA in cells has been shown to correlate best with the presence of e antigen in hepatocytes, core antigen in serum and histological cirrhosis (6). The presence of HBV DNA in large quantities has been shown in tissues with chronic active hepatitis when compared with adjacent areas of hepatocellular carcinoma (7).

The visualization of mRNA in tumour cells by ISH is used for the detection of specific gene expression, particularly that encoded by oncogenes and neuroendocrine genes. This is useful in typing the tumours. Predicting the prognosis of tumours has also been rendered possible as some of them are associated with amplification of oncogenes when they are aggressive. For example, increased N-myc expression is associated with poor prognosis in small-cell carcinomas of the lung. Non-small-cell carcinomas of the lung are associated with increased c-ras expression and breast carcinomas with c-erb expression (8,9).

ISH is probably the best nucleic acid probe technique for initial routine diagnostic applications. It has the advantage over other methods in that it permits unequivocal localization of HPV genomes to epithelial cells and thereby obviates the risk of false positive results due to laboratory or clinical contamination. Within the last few years various commercial kits have become available for the detection of HPV, EBV, CMV, adenovirus, hepatitis B virus, human immunodeficiency virus (HIV), *Candida albicans,* cryptococcus, etc. Nonradioactive biotinylated probes are more commonly used because of the advantages that have been previously mentioned. With a good positive and negative control, and thorough familiarity with the technique and the microscopical picture, a good result can be achieved. Because of all its advantages the ISH procedure has already secured a place in the laboratory as a reliable research and diagnostic tool.

Polymerase chain reaction

Since its development in 1985, the polymerase chain reaction (PCR) technique has had a major impact on medical research. There is no doubt that it is one of the most substantial technical advances in molecular genetics.

The PCR method, developed by a team of workers at the Cetus Corporation in Emeryville, California, is based on the repetitive cycling of three reactions, the conditions of which vary only in the temperature of incubation. All three reactions occur in the same tube and with temperature-stable reagents; the repetitive cycling is self-contained and fully automated. By synthesizing many copies of a selected DNA sequence, PCR is capable of substantially increasing the quantity of this target DNA segment in a sample. Enzymatic amplification is performed in discrete cycles and each cycle can, in principle, double the amount of DNA. The target is therefore exponentially amplified such that after n cycles there is $(1 + x)^n$ times as much target as was present initially (x represents the mean efficiency of each cycle). The technical advances which have made PCR a routine tool are (a) the use of a thermostable DNA polymerase which can withstand the high temperature required for DNA denaturation; (b) the production of microprocessor-controlled programmable heating blocks; and (c) the ready availability of synthetic oligonucleotides.

A target DNA sequence to be amplified is chosen first. Though it may not be necessary to know the nucleotide sequence of the target DNA, sequences of short stretches of DNA on either side of the target DNA must be known. These side sequences are used to design and synthesize two oligonucleotides known as primers. The two oligonucleotide primers are single strands of DNA which are made by an automated DNA synthesizer and are each approximately 20 nucleotides long. Each pair of primers is designed so that the nucleotide sequence of one primer is complementary to sequences flanking one end of the target DNA,

and the other primer is complementary to the other flanking sequence. The other two reagents that are important for the amplification process are large amounts of the four deoxyribonucleoside triphosphates and the heat-stable enzyme, Taq DNA polymerase, isolated from the thermophilic bacterium, *Thermus aquaticus.*

Each PCR cycle consists of three steps: DNA denaturation, primer hybridization and DNA polymerase extension. Each of these three steps must be carried out at an appropriate temperature. In the first step, the native double-stranded DNA is heat denatured. The target DNA melts, liberating single strands of DNA, which can subsequently reanneal to any other DNA that has complementary sequences. In the second step, which is performed at reduced temperatures, the oligonucleotide primers are annealed to complementary sequences on opposite strands of the target DNA. These two primers define the two ends of the amplified stretch of DNA. It is important that these two primers do not anneal to each other and their sites of annealing should be sufficiently distant from one another to allow the subsequent synthesis of new products. The specificity of PCR is due to the precision of this primer-template annealing.

The third step involves the synthesis of a complementary second strand of new DNA which occurs through the extension of each annealed primer by Taq polymerase in the presence of excess deoxyribonucleoside triphosphates. A new single strand of DNA is synthesized for each annealed primer. An important feature of the PCR is that all previously synthesized products act as templates for new primer-extension reactions (i.e. DNA synthesis) in each new cycle. After extension of the primers, the cycle is repeated, first by raising the temperature so that all double-stranded DNA is converted to single-stranded DNA, and then lowering the temperature to allow annealing and extension.

The whole process is taken through approximately 30 cycles. Because the quantity of target DNA theoretically doubles with each cycle, as few as 20 cycles generate approximately a million times the amount of target sequence present initially. One of the expected problems is the annealing of the primer to nontarget sequences, which lowers the purity of the target in the final product. The extent to which imperfect annealing and extension can occur depends on the temperatures during the primer annealing and polymerase extension steps, because the specificity of primer annealing is greater at higher temperatures. The use of a heat-resistant DNA polymerase that allows annealing to be carried out at an elevated temperature reduces this problem.

The specific amplified DNA sequences can be identified by different methods such as (a) direct visualization by fluorescence on agarose or polyacrylamide gel after staining with ethidium bromide; (b) radioactive-labelled probes; (c) nonisotopic colorimetric methods using enzyme-labelled probes; (d) probes bound to membranes; and (e) luminescent probe detection. The commonest method of detection of PCR products is by ethidium bromide staining following gel electrophoresis. Silver staining is also being used in chemical cleavage mismatch detection and single-strand conformation polymorphism.

One advantage that PCR has over many DNA probe diagnostic methods is that even a small amount of degraded or damaged DNA can serve as a template for the first cycle of amplification. Because subsequent cycles mainly use the newly synthesized product of previous cycles as template, poor quality samples do not create problems once amplification is underway. An important implication of the high sensitivity of PCR is that patient sampling requirements can be more convenient or less invasive. For example, buccal epithelial cells derived from a mouthwash have been used to identify carrier status for a cystic fibrosis mutation. Urinary sediments can be used in place of urethral swabs in the diagnosis of infectious urethritis and peripheral blood may be used instead of bone marrow or liver biopsy in the diagnosis of atypical mycobacterial infections.

Problems associated with PCR include sample contamination which can result in false positives with DNA amplification methods. Sample-to-sample contamination may occur when a positive specimen contaminates a negative one during sample preparation or during the procedure. Another possible source

of sample contamination is from nonviable organisms, previously grown or prepared in the sample preparation area. Pipettes that have been used on amplified DNA must never be used for isolation or aliquoting of sample DNA. False positive results can also occur at the detection stage, e.g. when the liquid transfer device pipettes a strong positive sample followed by a negative sample. To avoid this, positive displacement pipettes or tips that prevent aerosols from contaminating the device should be employed. A third possible contamination involves the inadvertent contamination of a new reaction with the aerosolized products of a previous reaction. Recommended precautions involve the use of positive displacement pipettes and the physical separation of areas where PCR reactions are analyzed from those where new reactions are set up.

Because of its ability to amplify a segment of the DNA of choice to a million more copies in a short time, mutations in genes or sequences indicative of an infectious agent can be identified more easily with a high degree of sensitivity. Many standard cloning methods have been supplemented by PCR. PCR has been useful in clinical medicine particularly in the diagnosis of infectious and neoplastic diseases, prenatal diagnosis of genetic disorders, and HLA analysis and tissue typing for organ transplantation. Because of its high sensitivity the definition of 'minimal residual disease' has been reviewed. It has also provided further insights into the prognostic variables and early relapse of certain malignancies (10).

In the field of infectious diseases, PCR has been extensively used in the identification of viruses, fungi and bacteria, especially those that are difficult to culture (Table 1). Thus, in many instances it has led to earlier diagnosis and appropriate medical treatment. Subtyping of HPV has been facilitated by PCR. Molecular fingerprinting has also been used for epidemiological studies to trace infections from a common source, a useful tool for hospital infection control efforts (10).

TABLE 1

VIRUSES, BACTERIA AND PARASITES THAT HAVE BEEN DETECTED BY PCR

Viruses	**Bacteria**	**Parasites and fungi**
Hepatitis A, B and C virus	*Chlamydia trachomatis*	*Cryptococcus neoformans*
Cytomegalovirus	*Chlamydia pneumoniae*	*Histoplasma capsulatum*
Epstein-Barr virus	*Mycoplasma pneumoniae*	*Coccidioides immitis*
Herpes simplex virus	*Rickettsia rickettsi*	*Pneumocystis carinii*
Human papillomavirus	*Mycobacterium leprae*	*Toxoplasma gondii*
Parvoviruses	*Mycobacterium tuberculosis*	*Trypanosoma brucei*
Rotaviruses	*Borrelia burgdorferi*	*Trypanosoma cruzi*
HIV 1 and 2	*Treponema pallidum*	*Plasmodium falciparum*
HTLV I and II	*Escherichia coli*	
Enteroviruses	*Shigella*	
Rhinoviruses	*Bordetella pertussis*	
Influenza viruses	*Clostridium difficile*	
Mumps virus	*Streptococcus pneumoniae*	

HIV human immunodeficiency virus
HTLV human T-lymphotropic virus

Application of PCR to monitor therapy for diseases such as hepatitis C and AIDS appears promising. In Papua New Guinea where malaria is endemic Felger et al. (11,12) have developed a rapid technique for MSA2 genotyping of *Plasmodium falciparum* from infected individuals. The technique used was a combination of PCR and restriction fragment length polymorphism (RFLP), referred to as amplified fragment length polymorphism. PCR was used in the first step to generate sufficient sample for analysis and the amplified PCR product was then subjected to genotyping using RFLP analysis. Several workers have used PCR to detect *P. falciparum* parasites in human blood specimens (13,14). At the Papua New Guinea Institute of Medical Research, Goroka, PCR is being used in the detection and genotyping of *Chlamydia trachomatis* from clinical samples from pregnant mothers, their newborns and their male partners. The samples include cervical scrapes, throat and conjunctival swabs, nasopharyngeal aspirates and urines (C.S. Mgone, personal communication).

PCR has been applied to the diagnosis of chronic myeloid leukaemia, through the detection of a bcr/abl translocation, even when it is present in only one of a million cells, results being available in as little as one to two days. In these patients bcr/abl translocations may be detected by PCR for several months following bone marrow transplantation without being associated with early relapse. Methods that permit extrapolation of PCR results appear to be the most clinically relevant indicators of relapse (10).

PCR has made it possible to differentiate between monoclonal and polyclonal lymphocytic proliferations, providing the basis for differential diagnosis between lymphomas and nonneoplastic lymphoid proliferations. The study of the rearrangement of beta-receptor genes of T cells and immunoglobulin genes of B cells by this technique forms the basis of the differential diagnosis of T and B cell lymphomas. The 8:14 translocation in Burkitt's lymphoma has similarly been studied using PCR.

Certain malignancies are associated with loss of genes, these being referred to as suppressor oncogenes or antioncogenes (15). The identification of these oncogenes has been facilitated by PCR.

Retinoblastoma was the first tumour in which the loss of a suppressor oncogene was demonstrated, lending support to Knudson's two-point theory of carcinogenesis. A loss of band 14 on the long arm of chromosome 13 was demonstrated in this tumour. Other examples of tumours with deletions of genetic material related to suppressor genes include familial adenomatous polyposis, colonic cancer, Wilm's tumour, and lung and bladder cancers (16). Gene amplification in tumours has been shown to be associated with aggressive behaviour. The N-*myc* oncogene in neuroblastoma (17) and HER-2/*neu* in breast and ovarian carcinoma (18) are some of the examples of gene amplification association with aggressive tumour behaviour. These amplifications, deletions and other alterations in the genes have been well demonstrated with hybridization techniques utilizing PCR.

PCR is extensively being used in the study of hereditary diseases. It has played a key role in delineating the molecular pathology of Melanesian (South-East Asian) ovalocytosis, a polymorphism found in Papua New Guinea where malaria is endemic. In this condition red cells resist invasion by malaria parasites; this has been attributed to the greater rigidity of ovalocytic red blood cells. The abnormality results from the heterozygous presence of an altered transmembrane protein (band 3). PCR has shown the presence of a 9-aminoacid deletion of the sequence AFSPQVLAA. This deletion is associated with an abnormal band 3 whose membrane domain has an altered structure and which is defective in anion transport activity (19,20). Highly divergent molecular variants of human T-lymphotropic virus type I have been detected from isolated populations in Papua New Guinea and the Solomon Islands using DNA sequencing techniques in PCR-amplified material (21). Similarly, Chen et al. (22) used PCR to study the New Guinea C dengue-2 virus nucleic acid sequences.

PCR has been found useful for evaluative purposes in the detection of the human immunodeficiency virus (HIV) genome in people who cannot be determined to be HIV positive by conventional means, for example,

infants born to HIV-infected mothers and seronegative people at high risk for the acquired immune deficiency syndrome. The clinical applications of PCR for the diagnosis of viral infection include the detection of neonatal infection, early infection, resolution of indeterminate serologies, viral typing, differentiation of indigenous viruses and vaccine strains, and identification of new agents. Table 1 lists some of the viral diseases that can be diagnosed with PCR.

Because culture is the 'gold standard' diagnostic method for most bacterial infections, the greatest potential opportunity for PCR to contribute to clinical medicine is in detecting pathogens that are slow or fastidious. Table 1 lists bacterial pathogens that can be detected by PCR. Many of these tests are still to be routinely used in clinical laboratories. PCR may also be useful in antibiotic susceptibility testing. Concordance between the presence of various genes for antibiotic-modifying enzymes and bacterial susceptibility in vitro has recently been demonstrated (23). PCR is also beginning to have an impact on the diagnosis of fungal and parasitic infections (Table 1). Universal fungal primers and pathogen-specific probes promise rapid and sensitive diagnosis of fungal sepsis and pulmonary disease.

A related concern for some applications is how to interpret the significance of results when PCR is far more sensitive than previous methods. It may be necessary to carry out epidemiological studies to ensure that the infectious agent is not much more widely prevalent in the general population than had been originally thought.

It is clear that nucleic acid amplification techniques, of which PCR is an example, will have a substantial future impact on the practice of laboratory medicine. Amplification techniques have already become the standard for most genetic disease tests and are becoming important for many infectious agents, especially as automated equipment becomes readily available to reduce costs and turnaround times.

Conclusion

Molecular biology techniques are being widely used in the modern-day practice of medicine in developed countries. They have become the established tools of diagnosis of disease conditions in clinical laboratories. They have transcended from research laboratories to diagnostic laboratories. In Papua New Guinea, these techniques are still at the level of research laboratories. It may be a long time before they are available in diagnostic laboratories for clinical use. It is, however, essential that practising clinicians are cognisant of the basics of these techniques and their applications. This knowledge will pave the way for a better understanding of disease processes at the molecular level. It is hoped that this paper and the two earlier papers in the series (24,25) will help to foster this better understanding and promote the wider use of these powerful new techniques in our clinical practice.

REFERENCES

1 **Brigati DJ, Myerson D, Leary JJ, Spalholz B, Travis SZ, Fong CK, Hsiung GD, Ward DC.** Detection of viral genomes in cultured cells and paraffin-embedded tissue sections using biotin-labeled hybridization probes. *Virology* 1983;126:32-50.

2 **Myerson D, Hackman RC, Nelson JA, Ward DC, McDougall JK.** Widespread presence of histologically occult cytomegalovirus. *Hum Pathol* 1984;15:430-439.

3 **Beckmann AM, Myerson D, Daling JR, Kiviat NB, Fenoglio CM, McDougall JK.** Detection and localization of human papillomavirus DNA in human genital condylomas by in situ hybridization with biotinylated probes. *J Med Virol* 1985;16:265-273.

4 **Wright RG, Murthy DP, Gupta AC, Cox N, Cooke RA.** Comparative in situ hybridisation study of juvenile laryngeal papillomatosis in Papua New Guinea and Australia. *J Clin Pathol* 1990;43:1023-1025.

5 **Nakamura S, Tourtellotte WW, Shapshak P et al.** The detection of papovavirus nucleotide sequences in frozen brain tissue from a patient with progressive multifocal leukoencephalopathy by in situ hybridization: comparison of in situ hybridization using isotopic and biotinylated probes. [Jpn]. *No To Shinkei [Brain and Nerve] (Tokyo)* 1985;37:359-364.

6 **Burrell CJ, Gowans EJ, Rowland R, Hall P, Jilbert AR, Marmion BP.** Correlation between liver histology and markers of hepatitis B virus replication in infective patients: a study by in situ hybridization. *Hepatology* 1984;4:20-24.

7 **Blum HE, Haase AT, Harris JD, Walker D, Vyas GN.** Asymmetric replication of hepatitis B virus DNA in human liver: demonstration of cytoplasmic minus-strand DNA by blot analyses and in situ hybridization. *Virology* 1984;139:87-96.

8 **Cline MJ, Battifora H.** Abnormalities of protooncogenes in non-small-cell lung cancer: correlations with tumour type and clinical characteristics. *Cancer* 1987;60:2669-2674.

9 **Funa K, Steinholtz L, Nou E, Bergh J.** Increased expression of N-myc in human small cell lung cancer biopsies predicts lack of response to chemotherapy and poor prognosis. *Am J Clin Pathol* 1987;88:216-220.

10 **Kaul K.** The impact of molecular pathology on disease diagnosis. *Lab Med* 1995;26:36-38.

11 **Felger I, Tavul L, Kabintik S, Marshall V, Genton B, Alpers M, Beck H.** *Plasmodium falciparum:* extensive polymorphism in merozoite surface antigen 2 alleles in an area with endemic malaria in Papua New Guinea. *Exp Parasitol* 1994;79:106-116.

12 **Felger I, Tavul L, Beck HP.** *Plasmodium falciparum:* a rapid technique for genotyping the merozoite surface protein 2. *Exp Parasitol* 1993;77:372-375.

13 **Felger I, Tavul L, Narara A, Genton B, Alpers M, Beck HP.** The use of the polymerase chain reaction for more sensitive detection of *Plasmodium falciparum. PNG Med J* 1995;38:52-56.

14 **Wataya Y, Arai M, Kubochi F, Mizukoshi C, Kakutani T, Ohta N, Ishii A.** DNA diagnosis of falciparum malaria using a double PCR technique: a field trial in the Solomon Islands. *Mol Biochem Parasitol* 1993;58:165-167.

15 **Cotran RS, Kumar V, Robbins SL, eds.** Neoplasia. In: Robbins' Pathologic Basis of Disease, 4th edition. Philadelphia: WB Saunders, 1989:289-291.

16 **Barlow SA.** Oncogenes and surgical pathology. *Semin Diagn Pathol* 1987;4:194-199.

17 **Brodeur GM, Seeger RC, Sather H, Dalton A, Siegel SE, Wong KY, Hammond D.** Clinical implications of oncogene activation in human neuroblastomas. *Cancer* 1986;58:541-545.

18 **Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL.** Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. *Science* 1987;235:177-182.

19 **Jarolim P, Palek J, Amato D, Hassan K, Sapak P, Nurse GT, Rubin HL, Zhai S, Sahr KE, Liu SC.** Deletion in erythrocyte band 3 gene in malaria-resistant Southeast Asian ovalocytosis. *Proc Natl Acad Sci USA* 1991;88:11022-11026.

20 **Schofield AE, Reardon DM, Tanner MJ.** Defective anion transport activity of the abnormal band 3 in hereditary ovalocytic red blood cells. *Nature* 1992;355:836-838.

21 **Gessian A, Yanagihara R, Franchini G, Garruto RM, Jenkins CL, Ajdukiewicz AB, Gallo RC, Gajdusek DC.** Highly divergent molecular variants of human T-lymphotropic virus type I from isolated populations in Papua New Guinea and the Solomon Islands. *Proc Natl Acad Sci USA* 1991;88:7694-7698.

22 **Chen HS, Guo HY, Chen HY, Liag YK.** Amplification of dengue 2 virus ribonucleic acid sequence using the polymerase chain reaction. *Southeast Asian J Trop Med Public Health* 1992;23:30-36.

23 **Narayan S.** Overview of principles and current uses of DNA probes in clinical and laboratory medicine. *Ann Clin Lab Med* 1992;22:353-376.

24 **Erasmus RT, Murthy DP, Ogunbanjo BO.** Basics of molecular biology and its applications: I. Molecular biology in medicine: basic concepts. *PNG Med J* 1996;39:56-66.

25 **Erasmus RT, Murthy DP, Ogunbanjo BO.** Basics of molecular biology and its applications: II. An outline of common laboratory techniques used in molecular biology. *PNG Med J* 1996;39:129-134.

BIBLIOGRAPHY

1 **Fenoglio-Preiser CM, Wolff M, Rilke F.** Nucleic acid probe techniques for the routine surgical pathology laboratory. In: Fenoglio-Preiser CM, ed. Progress in Surgical Pathology Vol 8. New York: Macmillan, 1988.

2 **Anthony PP, MacSween RNM, eds.** Recent Advances in Histopathology 14. Edinburgh: Churchill Livingstone, 1989, Chapters 1 and 2.

3 **Unger ER, Piper AM.** Nucleic Acid Probes. A Primer for Pathologists. American Society of Clinical Pathologists, 1989.