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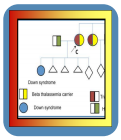
Table of Contents

GeNeDit 01
Common Genetic Disorders: Every Clinician's Responsibility

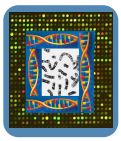
Clinical Vignette 02
More than one genetic affliction within the samefamily: Two case reports



GeNeViSTA 05
Current strategies for mapping the genes for Mendelian traits

GeNeViSTA 09
Application of Molecular Biology in Clinical Microbiology

GeNeXprESS 15
Next generation sequencing: window to a new era of molecular diagnostics



PhotoQuiz 17
PhotoQuiz 26

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Announcement

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Announcement

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Organized by: IAP Pune & Deenanath Mangeshkar Hospital, Pune

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GeNeDit

Common Genetic Disorders: Every Clinician's Responsibility

Editorial

Though we do not have prevalence data for most genetic disorders in India, sufficient prevalence data is available for at least some of the common genetic disorders such as beta thalassemia, Down syndrome and neural tube defects. The facilities for primary and secondary prevention of these common disorders are available in India and are rapidly increasing. At the same time, awareness about these disorders amongst clinicians and laypersons is also increasing. However, the level of facilities, knowledge of clinicians and awareness are markedly variable. There are no government-supported screening programs and neither are there any specific guidelines given by scientific or medical bodies. These facilities are not available at present in most of the medical colleges and hence, the approach to these common genetic problems is not being included in the practical training of medical students at the undergraduate or postgraduate levels.

In addition to prevention of birth of a first child with any of these genetic disorders, genetic counseling of the families with one child affected with a genetic disorder is very important. To identify a family at risk of a genetic disorder, a three-generation pedigree and screening of members of the extended family are important steps. These have been well illustrated in the two cases reported in this issue. Both the families had two genetic disorders in the family and due to timely genetic counseling and investigations, prenatal diagnosis could be offered to the at-risk pregnancies. In clinical genetics departments such coincidental occurrences of two genetic disorders in a patient or in a family are seen, though not commonly. Hence, it is important to offer screening for common genetic disorders to all families irrespective of the genetic disorder in the proband. As most of the patients with Down syndrome, thalassemia and neural tube defects are seen by pediatricians, they need to be confident about providing genetic counseling to the families and interpretation of the genetic tests. Similarly, obstetricians also need to play a more proactive role in the identification of families at-risk of genetic disorders by taking a detailed family history and offering screening tests. It is high time that every clinician be well conversant with pedigree drawing, basic genetics and principles of genetic counseling. Common genetic disorders can be easily tackled by most of the clinicians, especially those who are interested and have even had short term training in medical genetics.

This can partly happen by spreading basic knowledge about genetics applicable in clinical practice through short term training programs for clinicians at various stages of their careers. However, for future generations we need to impart an understanding of the clinical applications of medical genetics to undergraduate and postgraduate students. Training of committed medical college teachers from various specialties like pediatrics, obstetrics, pathology, anatomy, biochemistry, internal medicine, oncology, etc. and helping them to collaboratively set up genetic units in each medical college is the only way to take clinical genetics to all clinics. Training of teachers will have rapid, long term and multiplying effects. We need to take the fruits of research in genetics to the masses through clinicians from all specialties.

In this regard, I am glad to inform that the Indian Council of Medical Research has already initiated plans for creating a Network of Genetic Centers in medical colleges and I hope this initiative proves to be successful in achieving its goal of disseminating the knowledge of genetics to all medical practitioners.

Dr. Shubha R Phadke
1st October, 2014

More than one genetic affliction within the same family: Two case reports

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Genetic disorders, though rare, are still responsible for 2-3% of babies having congenital or genetically-determined abnormalities at birth. Rarer still is the possibility of more than one genetic disorder afflicting the same family. In spite of that, such families do exist and it is important that clinicians focus not only on the prevention of known genetic disorders in a family but also on screening for other common genetic disorders and prevention of these disorders in all families.

Case 1

A twenty-six year old female patient was referred by a gynecologist for pre-conceptional genetic counseling because her previous child had Down syndrome. Karyotype of the proband, which had been done previously, had shown Robertsonian translocation 46,XX,t(13;21). The couple was advised to get their blood karyotypes done. Along with that, as advised to all women who visit our medical genetics out patient department for preconception counseling or in the early antenatal period, we advised beta thalassemia carrier screening for the couple. The wife was found to be carrier of a balanced translocation, her karyotype being 45,XX,t(13;21) and the husband's karyotype was normal (46, XY). In addition, the couple found to be carriers for mutations in the *HBB* (beta globin) gene, the wife being carrier of the IVS 1-5(G→C) mutation for beta thalassemia and the husband being carrier of the HbE mutation. They were thus counseled about the risk of recurrence of Down syndrome and the risk of occurrence of beta thalassemia in their offspring and the possibility of prenatal diagnosis was also explained to them. The couple was also counseled regarding the importance of getting their extended family members screened for both the balanced translocation and thalassemia mutations running in their respective families. In line with the fair degree of understanding that the couple had acquired, three years later, the consultand's sister came to us with the concern of knowing the possibility of Down syndrome or thalassemia in her unborn child. She was not found to be a carrier of thalassemia mutation but was found to be carrying the balanced translocation (Fig 1). Amniocentesis and fetal karyotype was done for her at 16 weeks of pregnancy after due counseling. Karyotype of the fetus was 45,t (13;21) i.e. the fetus was carrying a similar balanced translocation as the mother. The family opted to continue the pregnancy.

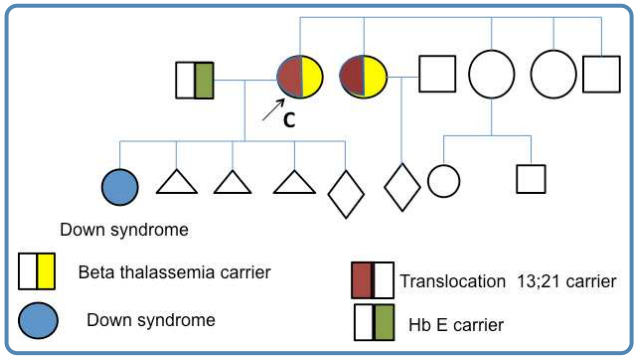

Figure 1 Pedigree of family 1.

Later, the parents of the proband came back to us at 10 weeks of pregnancy, for prenatal diagnostic testing. Chorionic villus sampling was done followed by karyotype of fetus and mutational analysis for beta thalassemia. The fetal karyotype showed 44 autosomes and 2 sex chromosomes. DNA mutational analysis for beta thalassemia revealed that the fetus was a carrier of the maternal beta thalassemia mutation. As the fetus was not affected

with either Down syndrome or beta thalassemia, the family opted to continue the pregnancy. Despite the rare situation of two genetic disorders being present within these two related families, both the couples were able to have healthy children after appropriate counseling and testing.

Case 2

A 28 year old woman presented to us at 6 weeks of gestation with history of a previous child being affected with transfusion-dependent thalassemia. She had been referred for prenatal diagnosis for the same in context of the present pregnancy. While talking to her 33-year old husband who had accompanied her to the OPD, it became apparent that he had subnormal intelligence. On further evaluation it was found that he had developmental delay and behavioral problems noticed since 3 years of age. He had never been to school, could not read, write or do simple calculations though he could take care of himself and helped in all the household work. The family history revealed presence of intellectual disability in the brother and maternal uncle and mild intellectual disability with behavioral problems in the mother and maternal aunt (Fig 2). In light of this family history suggestive of X linked inheritance, Fragile X syndrome was suspected. Southern Blot Analysis for Fragile X revealed increased triplet repeats in the FMR1 gene confirming our provisional diagnosis. Meanwhile, DNA mutation analysis of the *HBB* (beta globin) gene in the couple suggested that the husband was carrier for mutation of HbE and the wife was carrier for the IVS1-5(G-C) mutation of beta thalassemia. Both the mutations were confirmed in their previous affected child. So, there were 2 genetic disorders in this family, both of whose molecular bases were confirmed and hence prenatal diagnosis could be provided. Regarding intellectual disability, the family was counseled that the risk of fragile X in male offspring of the couple is negligible and all female offspring will be carriers of the mutation for fragile X. Carriers have variable severity of cognitive dysfunction that cannot be differentiated/ predicted by any DNA test. In this family there were two carrier females with some degree of manifestations. Targeted mutation analysis in chorionic villus sample collected at 11 completed weeks of pregnancy showed that the fetus was carrying both the thalassemia and HbE mutations like their previous child. The family opted for termination of the pregnancy.

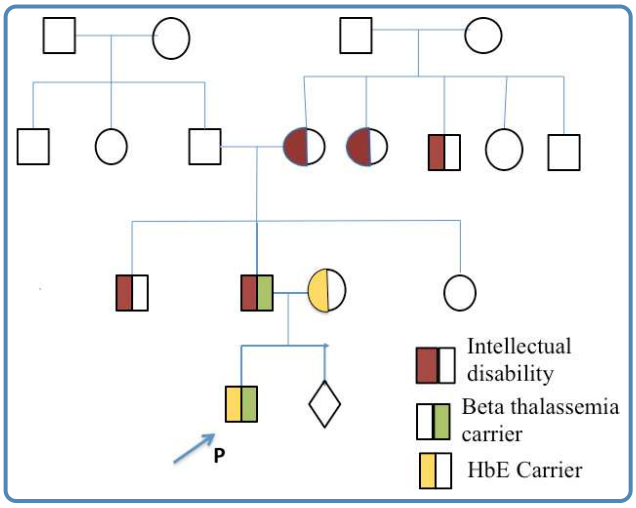

Figure 2 Pedigree of family 2.

Discussion

The first case emphasizes that the presence of a single disease should not divert attention from routine screening for other common genetic disorders. Beta thalassemia screening is advisable for each and every couple that is planning for childbirth because of the high carrier frequency of the disease in India.[1] High performance liquid chromatography of hemoglobin is an easy screening method for thalassemia which is widely available and the results of which are easily interpretable. Also noticeable and to be highlighted in this case is the importance of extended family screening for the already known genetic traits or diseases in the family. The second case again beautifully illustrates the fact that although genetic disorders are rare in occurrence, presence of more than one genetic disorder in the same family though less probable, is possible. With detailed and careful history, proper clinical evaluation and appropriate testing such families can be recognized and provided with the right course of medical action. It reiterates the fact that a family could have more than one genetic affliction and therefore we, as clinicians, should be vigilant enough to offer genetic counseling and appropriate investigations to the families at risk of a child with a serious genetic disorder or a disorder with handicap. These cases also stress the importance of pre-pregnancy genetic counseling, pedigree drawing and use of molecular and cytogenetic

investigations for genetic counseling and prenatal diagnosis.[2,3] This will only be possible with awareness about genetic investigations and counseling amongst primary care physicians, pediatricians and obstetricians.

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GeNeImage

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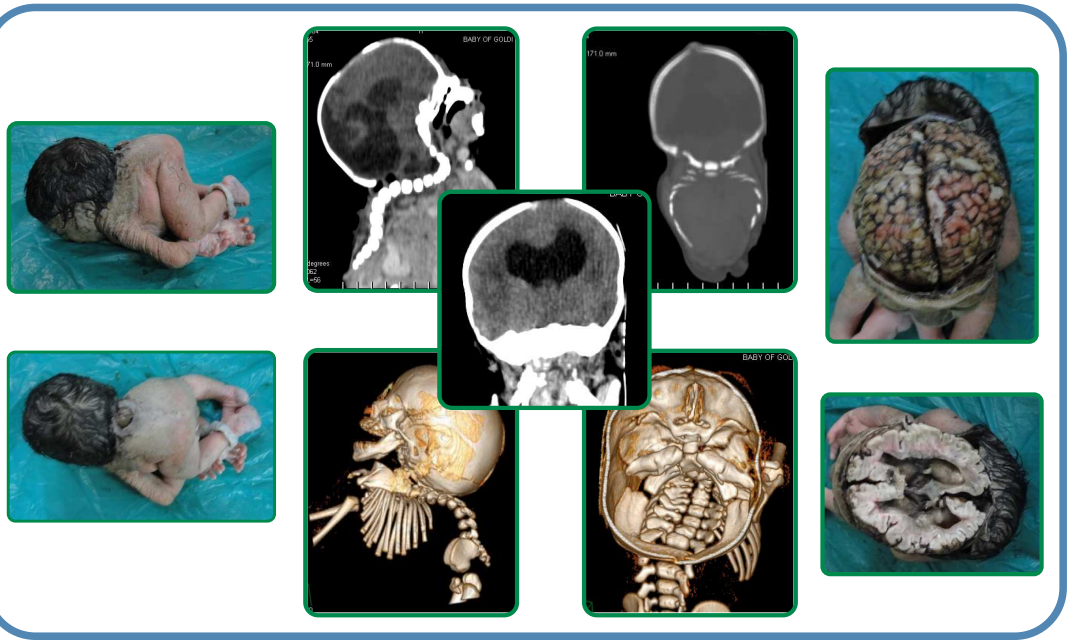
Iniencephaly, Spina Bifida, Holoprosencephaly and Polymicrogyria in a fetus

Iniencephaly is a malformation of multifactorial etiology. There is extreme hyperextension of the cervical spine resulting in face looking upwards and bone defect in occipital bone around foramen magnum. Encephalocele or spina bifida is usually present. Other anomalies like omphalocele, varying degree of holoprosencephaly, diaphragmatic hernia, cleft lip also may be present.



Current strategies for mapping the genes for Mendelian traits

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Mendelian traits or disorders refer to a group of phenotypes that exhibit one of the characteristic modes of inheritance: autosomal dominant, autosomal recessive and sex linked. These are also called 'single gene disorders' or 'monogenic traits' as it is usually a single gene that has a major effect on the phenotype. Though individually rare, as a group, there are more than 7,500 disorders known to be inherited in Mendelian fashion, and probably many more traits (normal variants) and so called private syndromes (affecting single families) exist. These are expected to affect about 5% of the general population. Hence they contribute to an important subgroup of human diseases and understanding them is important for any physician.

We now know that every nucleated cell of the human body has about 22,000 genes. At least 3,125 genes have been described to cause about 5,115 Mendelian phenotypes and we can expect many more genes to be annotated soon. Table 1 gives the current list of monogenic traits and the genes characterized (http://omim.org/statistics/entry accessed on 27 December 2013). We are likely to see the discovery of genes underlying single gene disorders progress exponentially over the next few years and some genes that cause private syndromes may then be characterized more slowly. This article reviews the current strategies that enable researchers to pin point a gene involved in the causation of a disease or phenotype.

Why should we map human diseases?

Understanding the genetic basis of human Mendelian disorders first of all provides an explanation for the phenotype. Simultaneously, this also enables us to understand the function of this gene in health or in other words the pathophysiology. Often a biological pathway in which the protein product is a component gets elucidated. This not only helps in understanding the causation of the disease, but also paves way for treatment of the condition as exemplified by the use of ivacaftor in cystic fibrosis caused by the G551D mutation in the *CFTR* gene and therapy for S447X mutation-related lipoprotein lipase deficiency.[1–3] In the clinic, this is translated to diagnosis, genetic counseling, predictive testing and prenatal diagnosis. The management is better guided by knowledge of the underlying genetic mechanism and preventive strategies can then be offered for the affected families.

Traditional gene identification strategies

Most of the traditional gene characterization strategies relied heavily on Sanger sequencing. Though it still remains the gold standard, next generation sequencing techniques have eased the burden on researchers. Candidate genes can be selected by the knowledge of the function of the involved protein (or similarity to a known protein function), a strategy called functional mapping. The more widely used positional cloning is discussed in the next section. An abnormal karyotype was often an important clue to the location of a genetic defect.[4] Routine cytogenetic analysis has taken a back seat with the entry of cytogenetic microarray, though even now we often resort to karyotyping in the clinic, when affordability is an issue.

Linkage analysis and positional cloning

Genome wide linkage analysis was first proposed in 1980.[5] This is one of the earliest and yet robust ways of identification of a gene for a Mendelian trait. Positional cloning simply refers to identification of the position of the gene along the human

chromosome and then selecting the specific gene for the disease and is probably the most successful approach.[6] Linkage assumes a specific mode of inheritance that often is inferred from the families selected for analysis. Several markers spread across the human genome are then typed and recombination events then define the boundaries of the position of the gene in question. Some of the successes of this approach lead to the discovery of genes for hemochromatosis, cystic fibrosis and Duchenne muscular dystrophy.[7–10] Cystic fibrosis is the most widely accepted example of early success of this approach.[8] Some other important milestones are identification of genes for lactose intolerance, chronic granulomatous disease, neurofibromatosis I, retinoblastoma and breast cancer.[11–16] Prior to the publication of the results of the Human Genome Project in 2003, it often used to be a mammoth task to clone these large segments of the genome before the gene could be identified. The Human Genome Project is now credited with making the information of all the genes in any region of the chromosome known for such a search and has accelerated the pace of gene discovery. If characterization of the first one thousand genes took two decades, the next decade saw more than 3,000 genes being identified.

Homozygosity mapping and autozygosity mapping

Autosomal recessive disorders often are precipitated by consanguinity. Identifying the regions of homozygosity in families affected with an autosomal recessive monogenic disorder can be an approach to identify the location of the gene.[17] The data from several families can be combined to narrow down the critical region to search for the candidate genes.[18,19] A similar strategy is autozygosity mapping that focuses on regions of homozygosity by descent in a single, usually large, family.[19,20] The current techniques of next generation sequencing and SNP microarray facilitate detection of the gene even in small kindreds and sporadic cases. Homozygosity mapping is the method of choice for gene mapping in the current era as the newer technologies require only a small number of affected individuals or families for research.[6]

Cytogenetic microarray

Cytogenetic microarray (CMA) is an important tool in the evaluation of individuals with intellectual disability and multiple congenital anomalies. Many sporadic or dominant Mendelian traits are known to be associated with copy number variations. The use of cytogenetic microarray has often led to further evaluation of the locus for a causative gene as illustrated by the thrombocytopenia-absent radius (TAR) syndrome.[21,22] CHARGE, once the best known example of an association, is now redefined as a syndrome after the identification of the causative gene.[23] CMA still remains an important tool for diagnosis in the clinic and for research by providing clues to the location of genes that cause Mendelian traits.[24]

SNP microarray in gene identification

The publication of a map of single nucleotide polymorphisms across the human genome has just made linkage analysis and homozygosity mapping easier than ever.[25] This has obviated the need for use of short tandem repeats as markers. The additional advantage is that the entire genotyping is now automated. The current platforms which often combine various oligonucleotide probes with SNPs make detection of copy number variants and linkage analysis possible in one experiment thus helping the clinicians in a dual way: in both diagnosis and research.[26]

Contribution of next generation sequencing techniques for gene mapping

Whole exome and whole genome sequencing have made gene discovery quicker and less expensive and have resulted in dramatic acceleration of gene identification in the last two to three years. The successful sequencing of human exomes was first reported in 2009 and the first identification of a gene for a Mendelian trait in 2010.[27,28] The same group further identified the gene *MLL2* for Kabuki syndrome that resided beyond what was perceived as the exome then.[29] Whole exome sequencing has now emerged as both a gene discovery and a diagnostic tool.[30,31]

Several paradigms or filters can be used in combination with the next generation sequencing

strategies to maximize the yield.[32] These include analysis of linkage, homozygosity, *de novo* occurrence of mutations and candidate genes.[33–35]

Whole exome sequencing has not only added tremendous pace to the discovery of genes for Mendelian phenotypes but also the variety of ways in which it can be applied. As illustrated by several researchers, this technique can be used to identify the gene for mosaic conditions like megalencephaly-capillary malformation syndrome and Proteus syndrome.[36,37] This strategy can also identify more than one gene, often involved in the same pathway, in a cohort of patients with similar phenotypes.[38–40] A recessive gene for Charcot-Marie-Tooth disease was identified from a single family and a gene for mental retardation could be identified from sporadic cases using this technique.[41,42] It has been successfully used as a diagnostic as well as a research tool in intellectual disability and to identify the genetic basis of novel syndromic mental retardation.[43,44]

Non–traditional strategies

Often sheer brilliance in analysis of a phenotype can identify the genetic basis of disease. *TRPV4* was postulated as a candidate gene for metatropic dysplasia by Ralph Lachman and his co-workers and was tested and confirmed because of the resemblance of the radiological features of this condition with spondylometaphyseal dysplasia, Kozlowski type.[45] Based on the phenotype in mouse models, researchers have shown that the genetic basis of human diseases can be identified.[46] Earlier knowledge of pathogenesis or components of a pathway was also used in identification of the *SMC1* gene for Cornelia de Lange syndrome.[47] The known gene *NIPBL* mediates its action through sister chromatid cohesion, of which the *SMC1* gene is also a component.

Confounding factors in gene mapping

Almost all gene mapping strategies rely heavily on exact phenotyping in the clinic. The selection of patients and families is very critical for the success of linkage which assumes that the phenotype is defined accurately. A detailed pedigree should be drawn and all possible modes of inheritance should be taken into consideration. Due consideration should be given to gonadal mosaicism and occurrence of new sporadic mutations. In addition, biological variations like reduced penetrance and variable expressivity of the mutation can be important confounding factors. Often, the phenotypes need to be re-examined to verify the accuracy. Etiological heterogeneity also needs to be kept in mind as some diseases that appear to be genetic may just be multifactorial (with genetic predisposition contributing only to a fraction of the phenotype) or environmental or teratogenic in causation.

DNA banking

A repository of human phenotypes with information on pedigree and DNA from the affected and unaffected family members has proven to be a vital part of gene discovery strategies. This way, collaborators across the world can share the clinical information and biological material to put together larger numbers of families for confirmation and validation of results and establish the causation. Once the genes for common Mendelian disorders are identified, these repositories will only gain more importance to identify the genetic basis of left-over private syndromes that occur only in one or two families or individuals. It is important that ethical implications of such DNA banking are given due importance to prevent misuse of such an effort.[48]

Current national and international scenario

Several efforts are underway to map the genes for the Mendelian traits. These include Finding of Rare Disease Genes (FORGE) in Canada, International Rare Disease Research Consortium in Europe and the Centers for Mendelian Genetics in United States.[49] It appears that it may take just a few years to identify most of them, as pointed out recently (the 'Mendeliome').[50] It is not surprising that an issue of a journal often carries articles on the discovery of a gene by two independent research groups, as for Cornelia de Lange syndrome and opsismodysplasia.[51–54]

India, with its huge population and practice of inbreeding in some select regions and communities, is a rich source of genetic material for research in this area. It is also likely that some of the Mendelian diseases manifest as 'private syndromes' in one or

only a few families. Though several centers now have the equipment, we are yet to see good collaborations that can be successful in identifying many genes. Hopefully the wait is not too long!

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GeNeViSTA

Application of Molecular Biology in Clinical Microbiology

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Introduction

During the past 10 to 15 years, there has been extensive growth in the use of molecular biology techniques in the clinical laboratory and their various applications. As a result of this development, many laboratories are able to offer increased sensitivity of testing, faster turnaround times, and ultimately improved patient care.[1] The applications of molecular technology in clinical microbiology are enormous; some of these applications include:

1. Early detection and identification of pathogens from clinical specimens and cultures
2. Classification of micro organisms based on their genetic relatedness (genotyping)
3. Detection of antibiotic resistance and toxin production
4. Detection of fastidious, slow growing or small numbers of pathogens in clinical specimens
5. Finding the host and agent factors conferring susceptibility, protection or virulence.

Conventional methods of pathogen detection

They include microscopy, culture and immunological reactions. Microscopy with Gram staining from the specimen gives the first indication of the suspected organism. Some other techniques are utilized in other cases like hanging drop preparation to look for highly motile bacteria in cases of suspected cholera, Ziehl - Nielsen staining for acid fast bacilli in cases of pulmonary tuberculosis or split skin smears for cases of Hansen's disease. Microscopy is also utilized for rapid detection of parasitic infections like malaria, filaria and intestinal parasites. Culture and biochemical reactions are done subsequently for identification and speciation in cases of cultivable organisms. The time for culture varies from days to weeks; there has been advent of new automated culture and identification systems which have reduced this time to some extent.

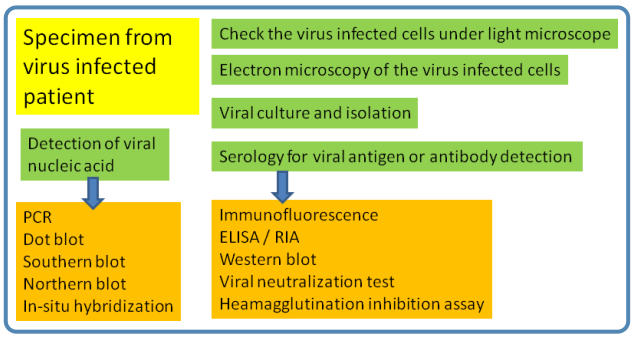

Figure 1 Processing of specimen for virological diagnosis (ELISA - Enzyme linked immunosorbent assay; RIA - Radioimmunoassay).

Microscopy has very limited usage in case of viral infections where serology and molecular techniques are the mainstay of diagnosis. After getting the specimen for a suspected case of viral infection it is further processed according to the schema shown in Figure 1, depending on the type of virus. For viruses such as Rubella and Hepatitis A, the onset of clinical symptoms coincides with the development of antibodies; here the detection of IgM antibody or rising titres of IgG antibody in the serum of the patient would indicate active disease. Some viruses produce clinical disease months or years after seroconversion e.g. human immunodeficiency

virus (HIV) and rabies virus. In the case of these viruses, the mere presence of antibody is sufficient to make a definitive diagnosis.

Viral cultures are done only at specialized centers. Viral culture techniques are less sensitive in some cases due to the low viral burden especially in encephalitis and also the presence of host neutralizing antibodies.

Types of specimen collected for microbiological studies
1. Respiratory tract infections: nasal and bronchial washings, throat and nasal swabs, sputum
2. Eye infections: throat and conjunctival swab/scraping
3. Gastrointestinal tract infections: stool and rectal swabs
4. Vesicular rash: vesicle fluid, skin scrapings
5. Maculopapular rash: throat, stool, and rectal swabs
6. CNS infection: stool, tissue, saliva, brain biopsy, cerebrospinal fluid
7. Genital infections: vesicle fluid or swab
8. Urinary tract infections: urine
9. Blood-borne infections: blood

Table 1 Types of specimen collected for microbiological studies.

Disadvantages of conventional methods

The gold standard in bacteriology largely remains culture, primarily due to cost factors and the complex nature of infections but there could be shortcomings like:

1. Minute quantities of pathogen may not be detected
2. The use of antibiotics before specimen collection reduces chances of pathogen detection
3. Some pathogens are difficult to culture, identify, or less amenable to susceptibility testing with conventional methods.
4. Need for speed – sometimes there is a need for timely intervention but the laboratory results of etiologic diagnosis take time

Similarly there could be limitations in cases of serological tests like:

1. Many viruses often produce clinical disease before the appearance of antibodies such as viruses causing respiratory and diarrhoeal diseases; hence, serology is not useful in these cases
2. Long length of time required for diagnosis for paired acute and convalescent sera
3. Mild local infections such as genital herpes may not produce a detectable antibody response
4. Antigenic cross-reactivity between related pathogens e.g. Herpes simplex virus (HSV) and Varicella zoster virus, Japanese B encephalitis and dengue, may lead to false positive results
5. Immuno-compromised patients often give a reduced or absent antibody response
6. Patients with infectious mononucleosis and those with connective tissue diseases such as SLE may react non-specifically giving a false positive result
7. Patients given blood or blood products may give a false positive result due to the transfer of antibody

Molecular methods for pathogen detection and quantification

There are a large number of molecular techniques which can be used for detection and quantitation of pathogens.[2] Some of these techniques include:

- Nucleic acid amplification: Nucleic acid amplification includes not only polymerase chain reaction (PCR) and its variants, but also alternate technologies, such as strand displacement amplification and transcription-mediated amplification. The technique involves selecting a specific genomic target in the pathogen of interest and designing specific primers for amplification of this target using Taq polymerase. Presence of a band of amplification of a specific size would indicate presence of the organism (Figure 2).

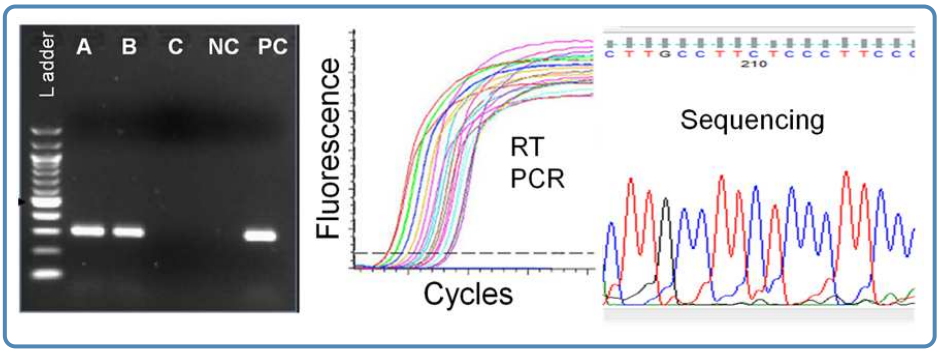

Figure 2 Examples of various molecular techniques used in clinical microbiology (Lanes A and B show presence of target nucleic acid, PC- positive control, NC- negative control).

- Real Time PCR: Real time PCR is mainly used as a method for quantification of viruses. Here the amount of amplification is checked at every cycle of PCR by incorporation of fluorescent dyes in the PCR mix or by designing fluorescent probes. The amount of fluorescence produced at every cycle is directly proportional to the initial quantity of the target nucleic acid (i.e. number of viral particles) (Figure 2).

- Sequencing: Molecular techniques come in handy when traditional phenotypic methods of microbial identification and typing are insufficient or time-consuming. Specific examples include the 16S rRNA sequencing for identification and subtyping of bacteria. 16S rRNA is a component of the 30S small subunit of prokaryotic ribosomes. The gene coding for it is referred to as 16S rDNA and is approximately 1500 nucleotides long. The 16S rDNA gene is highly conserved between different species of bacteria and archaea.[3] Viral genomes are relatively small, so they were among the first organisms to be fully sequenced. The complete DNA sequence of the Epstein-Barr virus was determined as early as 1984.

Applications of molecular techniques in clinical microbiology

The applications of molecular techniques in clinical microbiology depend on the organism being studied. The various applications are detailed below:

- For bacterial diseases: In case of fastidious bacteria like *Mycobacterium tuberculosis, Chlamydia trachomatis, Neisseria gonorrheae* and *Bordetella pertussis*, molecular testing has reduced the time taken by conventional culture to allow early detection and treatment. Although molecular methods have helped mycobacteriology, it is important to note that conventional culture still remains more sensitive. Despite this limitation, molecular detection of *M. tuberculosis* allows confirmation of acid-fast bacilli (AFB) seen on microscopy with up to 98% sensitivity within a day compared to approximately four weeks using phenotypic methods.

In the management of sexually transmitted diseases (STDs), traditional screening methods require invasive methods which are less acceptable as they cause embarrassment and discomfort, thus reducing compliance. Molecular methods offer more convenience and acceptance, enhancing the compliance. This has indirectly led to increase in laboratory confirmed cases of STDs. Molecular methods have the advantage of being performed on dry swabs with little degradation of the DNA (DNA is stable) during transit compared to the difficulties in maintaining viability during transport, which make them very useful for samples collected from remote and rural areas. In addition, molecular methods can detect multiple pathogens such as *C. trachomatis, N. gonorrhoeae, Haemophilus ducreyi* and the genital mycoplasma from the same swab.

Some bacteria can only be detected by molecular means as culture is either difficult or represents a significant occupational risk to the laboratory personnel. Examples include Whipple's disease due to *Tropheryma whipplei*, cat scratch disease due to *Bartonella henselae*, Q fever due to *Coxiella burnetii*, and male urethritis due to *Mycoplasma genitalium*. Molecular methods have the advantage here. In case of meningococcal disease, detection

can be done on the same day from specimens arising from sterile sites. Similarly, multiplex PCR methods have been developed for detection of other common bacterial causes of meningitis like *Streptococcus pneumoniae* and *Haemophilus influenza* type B.[4]

• For mycology and parasitology: Molecular testing can be helpful in certain circumstances. The diagnosis of *Pneumocystis jiroveci* pneumonia in immunosuppressed patients is limited to microscopy of respiratory tract specimens. Immunofluorescence is more sensitive than microscopy but is more expensive and needs specialized facilities. PCR can be useful but the specificity of PCR is limited because this organism is a ubiquitous commensal and can be detected in the absence of pneumonia. Another example is the use of 18S rRNA gene PCR to detect *Aspergillus* species infection in neutropenic patients.

In parasitological diagnosis, *Toxoplasma gondii* can be detected by PCR from amniocentesis fluid to confirm fetal infection and from cerebrospinal fluid (CSF) to diagnose toxoplasma encephalitis. Microscopy remains the mainstay of malaria diagnosis but *Plasmodium* species PCR, because of its superior sensitivity compared to microscopy, can diagnose malaria in patients whose thick and thin blood films test negative due to administration of chemoprophylaxis or partial immunity. *Plasmodium* species PCR can also detect mixed infections that can be difficult to detect microscopically.

• For viral diseases: a. Meningitis and encephalitis – The diagnosis of HSV encephalitis previously required brain biopsy in certain cases due to the low sensitivity of cerebrospinal fluid (CSF) culture and serology. PCR now allows the detection of HSV DNA from CSF with 95% sensitivity.[5] HSV PCR can be multiplexed with other pathogens responsible for meningitis.

b. Blood borne virus infection – Active hepatitis C viral (HCV) infections are diagnosed by the presence of HCV RNA, since the detection of antibody to HCV cannot distinguish between past and present infection. Early HIV infection and vertical transmission of HIV infection can be detected by the presence of HIV pro-viral DNA. Some blood banking services screen pooled samples from all donations for HIV and HCV using transcription mediated amplification assays, reducing the window period from 22 and 66 days to 9 and 7 days respectively.[6]

c. Genital /intrauterine infections - Cytomegalovirus (CMV), rubella, varicella zoster virus, and genital ulceration due to HSV type 2 infection are now routinely being detected by PCR.

d. Respiratory viral pathogens - Molecular detection of respiratory viral pathogens is cost-effective due to avoidance of unnecessary testing and hospitalization. It also helps reduce unnecessary antibiotic use. New assays of multiplex PCR are now available for testing all the common respiratory viruses along with fastidious bacterial causes of pneumonia. Uncommon viruses such as the severe acute respiratory syndrome- associated coronavirus (SARS-CoV) and influenza A/H5N1 (avian influenza) virus can also be incorporated into these tests.

e. Viral diarrhea - Viruses cause more infectious diarrhoea worldwide than bacteria and other pathogens. The method of choice for microbiological diagnosis of rotavirus from stool samples is PCR. Norovirus, responsible for large outbreaks both in the community and health care facilities is amenable for diagnosis by PCR, which is the most sensitive and rapid method. PCR is also the most sensitive method for the diagnosis of astroviruses and enteric adenoviruses.

• For treatment monitoring: Monitoring viral DNA or RNA load has become the standard of care for several chronic viral infections and is an integral component of the management of HIV, HCV and hepatitis B virus (HBV) infections. Measurement of the viral load is performed by real-time PCR. Patients who remain negative for HCV RNA 6 months after completing combination therapy for HCV infection almost always remain free of the virus.

• For sensitivity testing:

Bacterial pathogens: Detection of antibiotic resistance by molecular methods has become routine in many laboratories. This is done by detection of mutations in the organisms which confer resistance. Examples of genes studied include:

1. *mecA* gene in methicillin resistant *Staphylococcus aureus* (MRSA)
2. *vanA / vanB* genes in vancomycin resistant enterococci (VRE)
3. Extended spectrum β-lactamases (ESBL) in *Escherichia coli* and *Klebsiella pneumoniae*

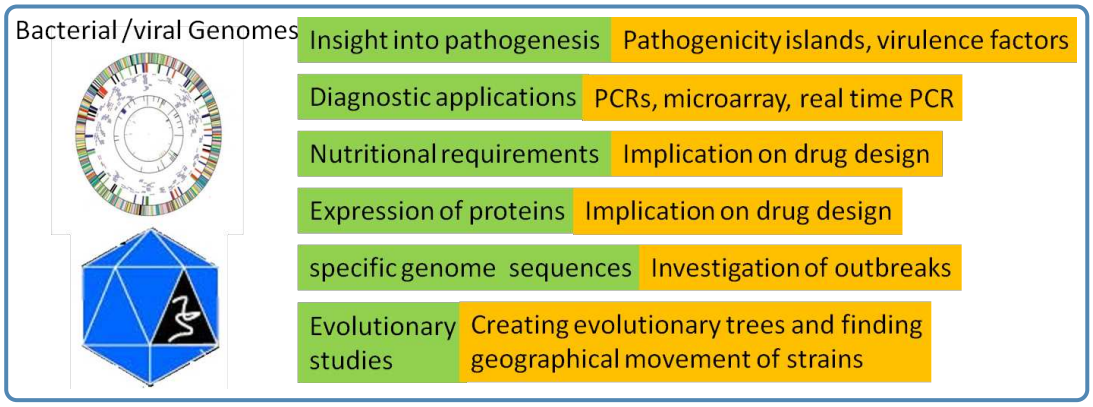

Figure 3 Applications of genome sequencing in bacteria and viruses.

4. Mutations in an 81-bp rifampin resistance-determining region (RRDR) in the *rpoB* gene in *M. tuberculosis*[7]

Summary of uses for molecular methods
1. Identification - nearly all possible pathogens
2. Viral load monitoring - Cytomegalovirus, Epstein-Barr virus, Hepatitis B, Hepatitis C, HIV
3. Viral genotyping - HIV, Hepatitis B, Hepatitis C, Human papillomavirus
4. Bacterial resistance detection - MRSA, VRE, ESBL producing E. coli, *K. pneumonia, M. tuberculosis*
5. Bacterial genotyping - *M. tuberculosis, N. meningitides*
6. Broad-range PCR - Infective endocarditis, bacterial meningitis

MRSA: Methicillin resistant staphylococcus aureus
VRE: Vancomycin resistant enterococcus
ESBL: Extended spectrum beta lactamase

Table 2 Summary of uses for molecular methods.

Viral pathogens: HIV genotyping for the detection of drug resistance is the standard of care to guide antiretroviral therapy and complements viral load assessment. Standard sequencing methodology and hybridization-based technology are the two principal methods used for HIV-1 genotyping. Genotyping is also critical to the management of chronic viral hepatitis. There are six HCV genotypes prevalent; of these, genotype 2 or 3 HCV infections have higher response to therapy compared to genotype 1 HCV infection.[8] Similarly, human papilloma virus (HPV) genotypes are also classified as either low or high-risk for the causation of cervical cancers.

Recent advances in pathogen discovery

• Microarrays in clinical microbiology: Microarrays using DNA chips have been extensively used for research, drug discovery and diagnostics. The most common use of DNA microarrays is for monitoring expression levels of transcripts from cells, viruses and bacteria in order to know which genes are being transcribed at a point of time. This method becomes useful to predict the function of uncharacterized genes or for analyzing the expression of virulence-associated genes (Figure 3). There could be extension of this application when response of a host to an invading pathogen is sought to throw light on the relation between host and pathogen as well as providing clues into the mechanisms of microbial pathogenicity.[9] Microarrays can be used to identify individuals who are more susceptible to infection and to determine prognostic markers for the outcome of the infection.

• Detection of new viral pathogens and vectors: This an upcoming application for which microarrays have potential. Here, an array containing the highly conserved sequences from fully sequenced reference viral genomes is designed. The aim is to detect a wide range of known viruses as well as novel members of existing viral families. The currently developed arrays include around 10,000

Limitations of Molecular Methods
1. Unlike bacterial culture, which can detect a large number of cultivable bacteria without initially knowing the specific organism, PCR can only detect the organism whose DNA is complementary to the primers used.
2. Differentiation between infection and disease, since the presence of nucleic acid does not necessarily mean the presence of viable microorganisms
3. Molecular tests are often subject to false positive results due to their high sensitivity
4. High level of staff training and skill is required for performing and interpreting these tests

Table 3 Limitations of Molecular Methods.

oligonucleotide probes capable of detecting almost 1,000 viruses in a single experiment. Another application for arrays could include the identification of distinct subspecies of vectors and reservoirs that harbour zoonotic pathogens (Figure 3).[10]

- Investigation of epidemic: Scientists in Taiwan identified and subtyped Influenza A virus as the H6N1 subtype, based on sequences of the genes encoding haemagglutinin and neuraminidase which were highly homologous to chicken H6N1 viruses. This virus had a G228S substitution in the haemagglutinin protein that might increase its affinity for the humans. The analysis was done in a few days as compared to long times needed for epidemic investigation by conventional methods.

- Revolution in bacteriology - identification of bacteria by Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF): There has been recent interest in bacterial identification using this technique which has been found to be rapid, inexpensive, and accurate. In one study 95.4% of the bacterial isolates were correctly identified in less than 1 hour. Of these bacterial isolates 84.1% were identified at the species level, and 11.3% were identified at the genus level.[11]

Conclusion

Molecular technology has gone beyond the era of research and has now become an integral part of any microbiological laboratory. The introduction of molecular methods in clinical microbiology laboratories not only depends on the performance of the test for each individual microorganism, but also on the clinical relevance of the diagnosis, the disease load and whether the new methods are supplementary to the procedures in use or their replacements. Therefore, strategies have to be devised for each infectious agent or clinical syndrome based on the available phenotypic information.

Acknowledgements

Dr. Ashish Bahal is a registered PhD student of Manipal University, Karnataka, India.

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Next generation sequencing: window to a new era of molecular diagnostics

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The advent of next generation sequencing has changed the scenario of diagnostic methodology. This new technique has been used in various areas ranging from the identification of new genes for single gene disorders, diseases with locus/ phenotypic heterogeneity, discovery of cancer causing genomic variations and understanding the pathogenicity of various microorganisms in human diseases.

Using single cell sequencing data to model the evolutionary history of a tumor[1]

Routine sequencing methods detect average signals generated by different cells within a tumor tissue. Single cell sequencing is a recently developed technology that detects genetic variation within a single cell and sequencing of different cells from a tumor can identify the cellular heterogeneity within a tumor. The analysis of mutations detected in different cells can identify the earliest mutation that is present in all cells and those that are present in further sub – clones. This helps to understand the sequential series of mutational events that resulted in a heterogeneous tumor. The major drawback of this technology is the high error rate. Kim and Simon have proposed a new statistical method that determines the order of mutation between any two sites and also reduces the error rate. They also provide a method to estimate the proportion of time from the earliest mutation event of the tumor to the most recent common ancestor (MRCA) of the cells sequenced. Based on the pair-wise mutation orders of all sites, the authors have constructed a mutation tree using the minimal spanning tree algorithm. The data was obtained by single cell exome sequencing of 58 cells from a patient with essential thrombocythemia (ET) and two tissue sequencing data (one normal and one tumor tissue). The process of carcinogenesis involves multistage progression of tumor clones, so identifying the order of mutation in various genes helps in better understanding of the tumor progression.

Whole–Exome Sequencing Identified KCNJ11 as the Thirteenth MODY Gene[2]

"Maturity onset diabetes of the young" is a heterogeneous disorder characterized by autosomal dominant mode of inheritance, onset before 25 years of age and a primary defect in the pancreatic β-cell function. Till now, 12 different types of MODY caused due to mutation in different genes have been identified and together they account for around 70% of the MODY cases and the remaining 30% are categorized as MODY-X. Bonnefond et al have done whole exome sequencing of four individuals (3 affected and one unaffected) from a large French MODY-X family. After analysis of the data they identified 324 variants of interest (present in all 3 affected individuals, not present in the unaffected individual and not reported in the database dbSNP130). The authors then assessed the status of these variants in an additional 23 members of the family and in 406 controls aged above 47 years with normal fasting blood sugar levels. Among variants that were not present in 406 controls, only one variant (c.679G>A; p.Glu227Lys) located in the KCNJ11 gene, at a heterozygous state was found to be present in all eight relatives with overt non autoimmune diabetes. Linkage analysis also suggested that KCNJ11 p.Glu227Lys mutation is causal for MODY in the analyzed pedigree. This mutation in KCNJ11 is already known to be associated

with neonatal diabetes mellitus and screening is currently indicated by guidelines in all patients who present with diabetes diagnosed before 6 to 12 months of age. As affected carriers of this mutation can be treated with sulfonylureas instead of insulin, this study suggests that molecular diagnosis of MODY should include KCNJ11 testing.

Lifting the lid on unborn lethal Mendelian phenotypes[3]

Hanan et al have shown, for the first time, the use of exome sequencing in identification of the causative genetic variation in embryonic lethal disorders. They studied a consanguineous couple with history of two fetal losses due to non-immune fetal hydrops. Fetal karyotypes in both the pregnancies were normal. In the current pregnancy, fetal hydrops was detected at 19 weeks gestational age with an otherwise normal ultrasound. So, considering consanguinity, the causal mutation was considered to be most likely homozygous. Exome sequencing of the fetus identified 4400 homozygous variants and out of them 440 were present within the autozygome. Out of these 440, 2 (R254C in CHRNA1 and P825S in SAP130) were novel, i.e not present in dbSNP and 250 controls. The mutation in CHRNA1 was particularly significant as this gene had been previously associated with multiple pterygium and fetal akinesia syndrome. The other was considered unlikely to be contributory, as mice homozygous for a transposon insertion in this gene lack similar phenotype. Thus, this study highlights the role of NGS in identification of the genetic cause of unborn lethal phenotypes.

Actionable Diagnosis of Neuroleptospirosis by NGS[4]

Approximately 50% cases of meningoencephalitis remain undiagnosed despite extensive clinical and laboratory evaluation. Because more than 100 different infectious agents can cause meningoencephalits, conventional diagnostic methods such as culture, PCR and serological tests can miss the diagnosis. The authors have proven the use of NGS for the identification of the infectious etiology in a 14 year old boy having severe combined immunodeficiency who presented with fever, headache, hydrocephalus and status epilepticus with history of minor illnesses for last 1 year. The workup for infectious diseases was negative. MRI of the head showed persistent hyperintensities in the basal ganglia and basilar leptomeningitis extending into the cerebral hemispheres. Biopsy of the right frontal lobe was performed and histologic examination revealed inflamed leptomeninges with a granulomatous infiltrate. Immunohistochemical testing and electron microscopy did not identify fungi, bacteria, or viruses. NGS of the cerebrospinal fluid and serum was done and bioinformatics analysis was done for the detection of all known pathogens which identified 475 of 3,063,784 reads of leptospira infection in CSF but not in serum. He was started on targeted antimicrobial therapy and gradually improved. Identification of leptospira infection was later confirmed by targeted PCR and Sanger sequencing. So, next generation sequencing has proven helpful in many clinical situations and its role has expanded from the identification of genes for known as well as uncharacterized Mendelian disorders to understanding the molecular genetic basis of cancers, common disorders and microbial infections.

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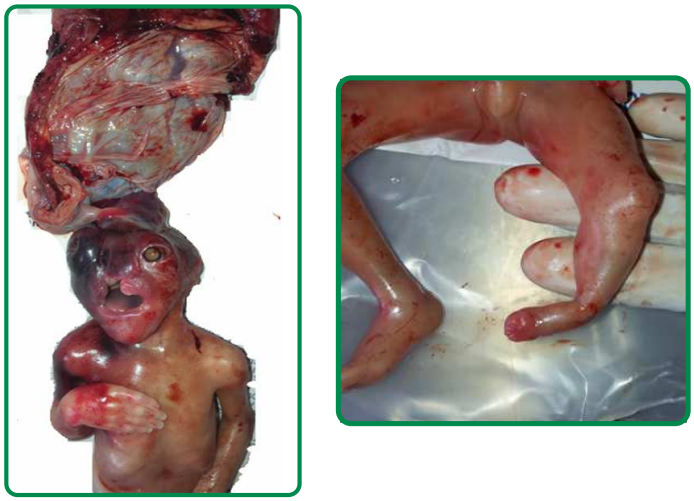
PhotoQuiz - 26

Contributed by: Dr. S J Patil
Narayana Hrudayalaya Hospitals, Bangalore.
Email: drsjpatil@gmail.com

This male fetus of 20 weeks gestational age was referred for fetal autopsy evaluation. Antenatal ultrasound had detected anencephaly and oral clefting. Identify the condition.

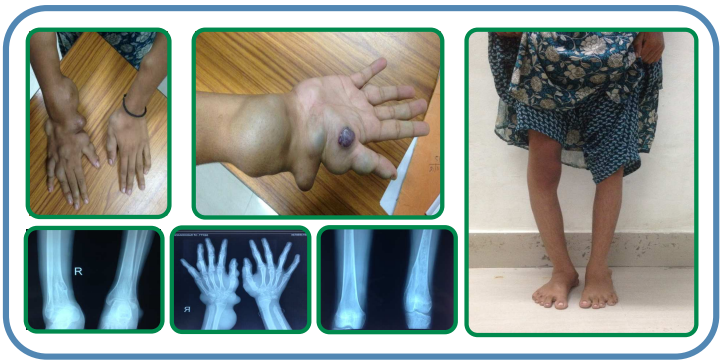
Please send your responses to editor@iamg.in
Or go to http://iamg.in/genetic_clinics/photoquiz_answers.php
to submit your answer.



Answer to PhotoQuiz 25

Maffucci syndrome (OMIM %614569)

Maffucci syndrome is a sporadic condition characterized by multiple hemangiomas and enchondromas. Apart from cosmetic concerns, enchondromas may lead to skeletal deformity and have potential for malignant change to chondrosarcoma.



Correct responses were given by:

1. Prashanth Verma, Saudi Arabia
2. Sreelata Nair, Adoor,Kerala
3. Sheetal Sharda, Chandigarh
4. Ravi Goyal, Kota, Rajasthan
5. Mohandas Nair, Calicut
6. Niby J Elackatt, Bengaluru
7. Saminathan D, Trichy
8. Sengodi E, Trichy
9. Thomas Gregor Issac, Bengaluru
10. Deepti Saxena, Lucknow

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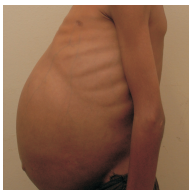
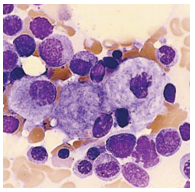
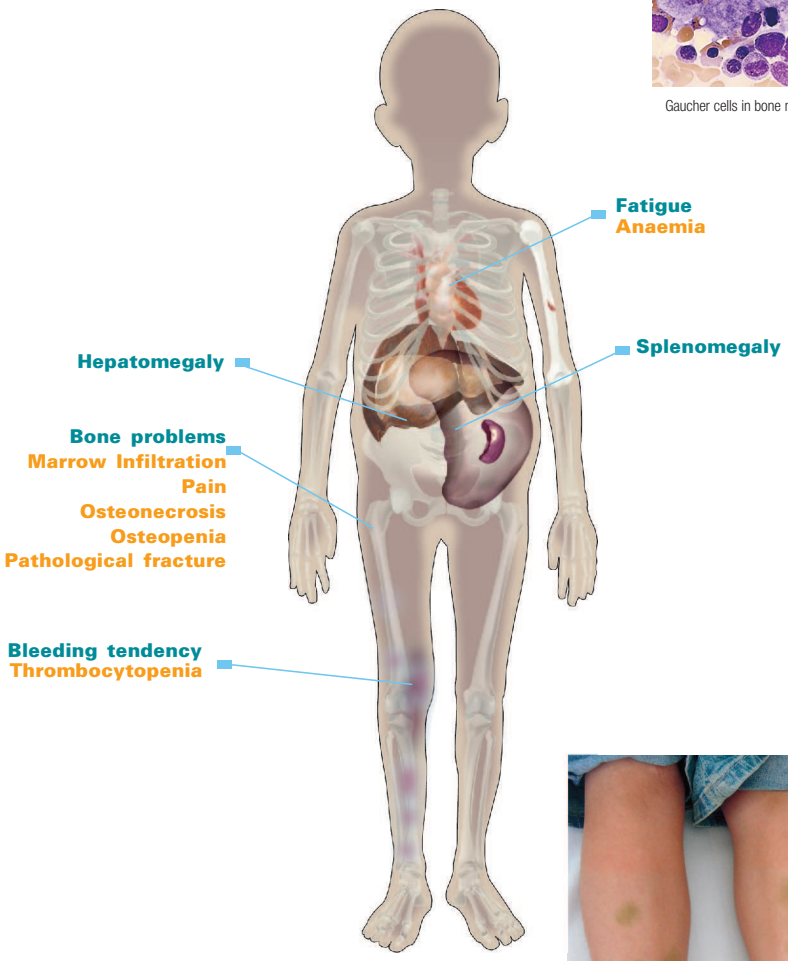


Enlarged Liver and Spleen



Gaucher cells in bone marrow



Hematoma[6]

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References: 1.Weinreb N et al. Clin Genet 2007 June; 71(6): 576-588. 2.Grabowski GA Lancet 2008 Oct. 372: 1263-1271. 3.Weinreb N et al. Am J Hematol. 2008 Dec. 83 (12): 896-900. 4.De Fost M et al. Blood Cells Mol Dis. 2006 Jan-Feb; 36 (1), 53 – 58. 5.Keutzer J Clin Therap 2009, 31, Supplement 3, Page S176. 6.Courtesy of Dr. E. Mengel

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