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# Recent advances in diagnosis of rheumatic heart disease

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**ABSTRACT**

*Methods for the diagnosis of infectious diseases have stagnated in the last 30-40 years. Few major advances in clinical diagnostic testing have been made since the introduction of PCR, although new technologies are emerging day by day. Many tests are based on very old and labor intensive technologies such as various biochemical assays (hippurate test, phadebact test, CRP), culture assays, antibiotic detection assays and imaging techniques. Now there is a need of more rapid tests without sacrificing sensitivity and specificity for the better results. In recent years, research has been focused on alternative methods to improve the diagnosis of rheumatic heart disease. These include immunoassay, molecular based approaches, DNA biosensor (amperometric molecular biosensor, graphene based DNA sensor, carbon nanotube based biosensor) and microarray techniques etc. This review summaries the progress the new approaches in diagnosis of rheumatic heart disease and highlights some of the merits and advantage of these tests.*

**Keywords:** *Biosensor, Pharyngitis, Rheumatic heart disease, Streptococcus pyogenes*

## INTRODUCTION:

Rheumatic heart disease (RHD is a condition in which heart valves are damaged as a complication of rheumatic fever. Rheumatic fever is an inflammatory condition which occurs in children and adolescents following pharyngitis by gram positive bacteria, Streptococcus pyogenes and thereby affecting many of the body's connective tissues especially those of the heart, joints, brain or skin etc. The most common symptoms of rheumatic infection are joint pain, fever, chest pain or palpitations caused by heart inflammation (carditis), jerky uncontrollable movements (Sydenham's chorea), a rash and small bumps (nodules) under the skin (Chandra *et al.,* 2009). Increasing resistance of *S. pyogenes* to antibiotics has called for the search of new preventive methods and therapies.

The adherence of bacteria to the host cells as the initial event in the pathogenic process is a potential target for anti-adherence therapy, in which analogues of receptor molecule are used to prevent the bacteria from binding to the host cells (Jukka *et al.,* 2001). The patient remains infected for weeks even after symptomatic resolution of pharyngitis and may serves as reservoir of infection to others. It is, therefore not only the therapeutic but also accurate detection and diagnosis of rheumatic heart disease infection is essential for prevention and clinical management of the disease. Most of the current tests rely on several laboratory methods in addition to clinical symptoms, clinical history and geographic location of the patient and are not useful for therapy and prognosis of the disease. Recent developments in new diagnostic tools, however, have open new avenues for vast improvements in the diagnosis of rheumatic heart infection. A number of newer serology based assays that are highly specific and sensitive have emerged, such as falcon assay screening test ELISA [FAST-ELISA] (Hancock *et al.,* 1986), Dot- ELISA (Pappas *et al.* 1984; Pappas *et al.,* 1988), rapid antigen detection system [RDTS] (Shokoples *et al.,* 2009) and luciferase immunoprecipitation system [LIPS] (Burbelo *et al.,* 2005).

Molecular based approaches such as loop mediated isothermal amplification [LAMP] (Parida *et al.,* 2008), real time polymerase chain reaction [RTPCR (Muldrew, 2009) have shown a high potential for use in diagnosis with increased specificity and sensitivity. iii) DNA biosensor and micro array technology have also been introduced for the discovery of biomarkers using tissues or biological fluids from the infected hosts. The present article describes the potential and efficacy of the existing technologies for diagnosis of rheumatic heart disease.

### Clinical manifestations and environmental factors for RHD

The spectrum of the clinical manifestations of the streptococcal infection may range from the unquestioned case to the more mild and difficult to be diagnosed clinically. As in case of a susceptible person a wide range of events can take place following streptococcal disease. The bacterium can be present in the nasopharynx without producing disease and thereby; showing no immunologic evidence of invasion

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into the tissues by the organism. In another situation, in the same person, the streptococci may invade the tissues, producing the usual symptoms of streptococcosis but not rheumatic fever (Forrest, 1955). However, in a significant proportion of susceptible persons, the streptococcal infection is followed by an unusual response of the heart tissue and cause rheumatic fever, which may be mild or severe.

Variation in strain virulence helps to account for the wide spectrum of group A streptococcal (GAS) diseases and for their striking epidemiological variation. Recent studies of the genetic control of the expression of the virulence factors of group A streptococci are beginning to illuminate such variation (Stollerman, 2002). Although, the pathogenesis of acute rheumatic fever (ARF) requires primary infection of the throat by highly virulent GAS strains (Gene, 2001). The streptococcal strains causing RF contain large hyaluronate capsules and extended M-protein molecules (Stollerman, 2002; Gene, 2003). The M-molecule contains some epitopes cross-reactive with host tissues, and also has super antigenic properties like the secreted GAS erythrogenic toxins (Charles, 1952). In settings where ARF has become rare, GAS pharyngitis continues to be quite common but is most often caused by relatively attenuated strains. These, however, may colonize the throat avidly, and often stubbornly. GAS "skin strains" that cause pyoderma (impetigo) is molecularly distinct from "throat strains". Although they may colonize and infect the throat, the pyoderma strains are generally less virulent and are not rheumatogenic.

Various environmental bacterial and host factors have been studied in relation to the occurrence of the rheumatic heart disease (Table 1). It is well established that the incidences of rheumatic fever may vary from year to year in the same population group (Antoni *et al.,* 1958). In general, rheumatic heart disease is most prevalent during winter and spring months at a time when respiratory infections reached their maximum incidence (Antoni *et al.,* 1958). Although the disease occurs frequently in the temperate zones but it can not be rare in all tropical areas. At a high altitude, under conditions of low humidity and less population, the incidences of rheumatic disease are higher. A greater risk of rheumatic fever is associated with the overcrowding, poor sanitation and other conditions that may easily result in the rapid transmission or multiple exposure to streptococcus bacteria (Hoby *et al.,* 2009).

**Pathogenesis of RHD**

The epidemiological association between group A hemolytic streptococcal infections and the subsequent development of acute rheumatic fever (RF) has been well established (Chopra *et al.,* 2007). RF is a delayed autoimmune response to group A streptococcal pharyngitis and the clinical manifestation of the response and its severity in an individual is determined by host genetic susceptibility, the virulence of the infecting organism and environment (WHO, 2001). Although, streptococci from serogroup B, C, G and F can cause pharyngitis and trigger a host immune response but it has not been linked to the etiology of rheumatic heart disease (WHO, 2004). There is considerable geographical variation in the prevalence of all serogroups of β-hemolytic streptococci.

In many tropical countries, up to 60-70% of isolates from the throats of asymptomatic children fall into serogroups C and G (WHO, 2001). Conversely, in temperate regions, serogroup A is more predominant isolate (50-60%) with serogroup C and G together accounting for less than 30% of the isolates. Non suppurative sequel, such as RF and RHD are seen only after group A streptococcal infection of the upper respiratory tract (Brahmadathan *et al.,* 2006). Post streptococcal glomerulo-nephritis may occur after an infection of either the throat or skin by nephritogenic stains of group A streptococci (Hugh *et al.,* 1979; Laura *et al.,* 2003). It is presumed that chronic streptococcal “carrier” states do not trigger the development of RF (Maria *et al.,* 2007).

It has been demonstrated that molecular mimicry between Streptococcus pyogenes antigen and human proteins leads to autoimmune reactions both humoral and cell mediated causing RF/RHD (Guilherme *et al.,* 2006). Heart tissues namely the valves, left atrial appendage (LAA) and myocardium reveal variable amounts of infiltration by lymphocytes. Significant endocarditis and valvulitis is observed in these cases (Dominik *et al.,* 2006). CD4 +T cells are most likely the ultimate effectors of chronic valve lesions in RHD (Fae *et al.,* 2004). They can recognize Streptococcal M5 protein peptides and produce various inflammatory cytokines such as TNF-alpha, IFN-gamma, IL-10, IL-4 which could be responsible for progressive fibrotic valvular lesions.

Cardiac myosin has been defined as a putative autoantigen recognized by autoantibodies of RF patients (Kellen *et al.,* 2006; Guilherme *et al.,* 2007). Cross reactivity between cardiac myosin and group A β-hemolytic Streptococcal M protein has been adequately demonstrated. The disease predominantly affects the valvular endocardium culminating in crippling valve deformities. Immune responses against cardiac myosin lead to valvular heart disease and infiltration of the heart by Streptococcal M-protein reactive T lymphocytes (Galvin *et al.,* 2002). Presence of inflammatory cells and increased expression of several cytokines in cases of "end stage" RHD reflects a possible subclinical, ongoing insult/injury to some unrecognized antigenic stimulus by β-hemolytic Streptococcal antigens that have sensitized

the various target tissues and which further culminate in permanent valve deformities.

**Diagnosis of RHD**

RF and RHD are the major health problems worldwide but especially in developing and poor countries (Atalar *et al.,* 2006). Accurate diagnosis is important not only for prevention and clinical management of initial episodes of the disease but also to decide the antibiotic therapy. The diagnosis of acute rheumatic fever may lead to the individual suffering a further attack of ARF, cardiac damage and premature death (Cardiac society, 2006). Diagnosis of ARF relies on health professionals being aware of the diagnostic features, particularly when presentation is delayed or atypical. The current approaches being in practice are discussed as below:

**Jones Criteria**

The Jones criteria for the diagnosis of ARF were introduced in 1944. The criteria divides the clinical features of ARF into major and minor manifestation, based on their prevalence and specificity (Mishra, 2007). Major manifestations are those that make the diagnosis more likely, where as minor manifestations are considered to be suggestive, but insufficient on their own, for a diagnosis of recurrent ARF.

The exception to this is in the diagnosis of recurrent ARF (Polly *et al.,* 2008). The Jones criteria have been periodically modified and updated. The 1992 update is currently the most widely used and quoted version. (Table 2). Each change was made to improve the specificity of the criteria instead of the sensitivity. The criteria may not be sensitive enough to pick up disease in high incidence populations An expert group of World Health Organization (WHO) has recently provided additional guidelines as to how the Jones criteria should be applied in primary and recurrent episodes, because the Jones and WHO criteria appear too restrictive (Jonathan et al., 2006).

**Microbiological Test**

Isolation and identification of group A β-hemolytic streptococci (GAS)

Laboratory diagnosis of streptococcal pharyngitis depends upon the successful isolation of β-hemolytic streptococci and its identification as GAS. This is carried out by culturing of bacterial species on to the sheep blood agar medium and incubation for 18h (Graham *et al.,* 1986). Further identification is confirmed by gram staining.

**Gram Staining**

Gram staining is a differential staining method of differentiating bacterial species into two large groups (Gram positive and Gram negative) based on the chemical and physical properties of their cell walls. It is additionally a critical test for the rapid presumptive diagnosis of infectious agents. The test was originally developed by Christian Gram in 1884, but modified by Hucker in 1921. Streptococcus pyogenes is a gram positive bacteria due to the presence of thick cell wall made up of peptidoglycan which stains purple, but this is not a sensitive method for the identification because some gram positive bacteria may lose the stain easily and therefore appear as mixture of gram positive and gram negative bacteria.

**Bacitracin Susceptibility**

In many cases bacitracin susceptibility test is the method of choice to identify GAS. This test has a sensitivity of >95% but it is not recommended since group G and C streptococci may give false
positive results. Batch to batch variation may occur in the commercial discs and therefore it is essential to test each batch for known GAS strain (Brahmadathan *et al.,* 2006). In this test streptococci form a clear zone around the discs and indicates the presence of GAS strain.

**Rapid Antigen Detection Test (RADT)**

Many GAS antigen detection tests are available commercially. Most of these tests have a high degree of specificity but their sensitivity are low in clinical practice. Therefore, treatment of patient is advised only with acute pharyngitis who has a positive RADT. As with the throat culture, a positive test may reflect chronic colonization by GAS and the acute illness may be caused by another agent (Michael *et al.,* 2009). With most RADT, a negative test does not exclude the presence of GAS and therefore throat culture should be performed. Some experts also believe that physicians who use an RADT without culture backup in children and adults should compare the results of RADT with blood agar plate cultures to confirm adequate sensitivity. Diagnosis of GAS pharyngitis in most adults on the basis of an RADT alone is not reasonable unless throat culture is not performed (Alan *et al.,* 2002).

**Streptococcal Antibody Tests**

Antistreptococcal antibody titers reflect past and not present immunologic events and therefore cannot be used to determine whether an individual with pharyngitis and GAS in the pharynx is truly infected or merely a streptococcal career. An elevated or rising antistreptococcal antibody titers provides indication of GAS infection in a patient of having rheumatic fever. The most commonly used and commercially available antibody assays are antistreptolysin O and antideoxyribonuclease B (Adnan *et al.,* 1995). These tests are valuable in patients who have possible non-suppurative complication of GAS infections. The anti-streptolysin O test is usually obtained first, and if it is

not elevated, an antideoxyribonuclease B test may be performed. Anti-streptolysin O titers begin to rise approximately in 1 week and may reach maximum in 3-6 weeks after the infection (Christhoper *et al.,* 2003). Antideoxyribonuclease B titers begin to rise 1-2 weeks and maximum in 6-8 weeks after the infection. Elevated titers for both tests may persist for several months after even uncomplicated GAS infections.
It is most common for physicians to misinterpret streptococcal antibody titers because normal levels of these antibodies are higher among school age children than adults.

The traditional antistreptolysin O and antideoxyribonuclease B tests both are neutralization assays. Other tests use latex agglutination or nephelometric assays. Unfortunately, these tests have not been well standardized against the traditional neutralization assays. Physicians need to be aware of these potential problems when interpreting the results of streptococcal serological test performed on their patients. A commercially available slide agglutination test, for the detection of antibodies to several streptococcal antigens, is the Streptozyme test (Alan *et al.,* 1974).This test is comparatively less standardized and less reproducible than other antibody tests, and it should not be used as a confirmatory test for GAS infection.

**PCR Based Diagnosis**
Molecular diagnostics are revolutionizing the clinical practice of infectious disease. The introduction of polymerase chain reaction (PCR) based DNA amplification in the early nineties used for diagnosis of infectious diseases. Lisa first time evaluated a PCR assay for detection of Streptococcus pyrogenic exotoxin B gene from tissue biopsy specimens of patients with necrotizing fasciitis (Marie *et al.,* 2002). Then Kaltwasser used polymerase chain reaction for the diagnosis of Streptococcus pyogenes by evaluating an optical immunoassay for the detection of group A streptococci in children with pharyngitis (Michael *et al.,* 2004).

Compared to serology, PCR-based diagnosis is more specific and sensitive and may allow early diagnosis of rheumatic heart disease. Adequate patient samples are important for PCR. Depending on the clinical setting, PCR amplification can be performed on swab samples, other clinical specimens or directly on histopathologic specimens. PCR based detection of pathogens DNA can be used to confirm serologic screening tests and to diagnose infections due to pathogens which are difficult to grow in culture. With chronically persistent agents, PCR may allow differentiation between clinically irrelevant and relevant infection (Tarr *et al.,* 2010). Kumar *et al* (2011) used PCR based diagnosis of group A streptococci using sof as a specific genetic marker.. Currently, in our lab PCR based diagnosis of rheumatic heart disease takes only 80 min to confirm the disease (Fig.1).

**Microarray**
DNA microarray technique is one of the latest advances in the field of molecular biology and medicines. It is a multiplex technique used in combination of bioinformatics and statistical data analysis. Since, 1995, the technique offers the possibility of conducting tens or hundreds of simultaneous hybridizations (Kumar, 2009). Microarray profiling offers many potential advances in diagnostic and therapeutic intervention in human disease. Lee and colleagues developed a DNA microarray called PathoChip for the detection of 44 highly prevalent and fastidious pathogenic bacteria. He used variety of clinical samples collected from blood, sputum, stool and urine to evaluate the technique. Another patented array composing of DNAs amplified with 35 kinds of primers used to amplify 16S and 18S sequences, specific antigen, toxin and virulent genes of pathogens causing respiratory infectious disease was developed by Ezaki in 2003.

Smoot *el al* (2001) has studied a comparative analysis of serotype M18 group A Streptococcus strains associated with acute rheumatic fever outbreaks using microarray technique (James *et al.,* 2002). Davignon has used resequencing oligonucleotide microarray for the identification of Streptococcus pyogenes with the benefit of sequencing information from microarray analysis.

**Future Diagnosis: Biosensors And Nanosensors**
Biosensor technology is an upcoming technology widely applicable in molecular diagnostics. Now a days biosensors and nano-sensors are of great research interest. There are many drawbacks in diagnosis of rheumatic infection by the above described methods. More diagnostic protocol has to be proposed to increase the sensitivity and selectivity of diagnosis.

Biosensors are generally defined as an analytical device which convert a biological response into a quantifiable and processable signal. The analytical devices are composed of the biological recognition elements which are directly interfaced to a signal transducer that converts biological signals into electrical, optical or other forms. DNA based biosensor has significant growth in recent years due to their potential applications for detection of nucleic acid and identification of microorganisms (Lixia *et al.,* 2011). Electrochemical DNA biosensors, which are based on

the nucleic acid hybridization, are now being more advance technique for diagnosis of pathogenic diseases.

Depending upon the probes employed, PNA (peptide nucleic acid) and DNA electrochemical biosensor have been developed for detection of pathogens. (Patel *et al.,* 2009) has developed an electrochemical biosensor for the detection of N. meningitidis. They had used thiol labeled specific probe for the hybridization with single standard genomic DNA (Patel *et al.,* 2009).

In recent years, with the successful development of nanotechnology, nanosensors for diagnosis of different diseases using nanoparticles such as carbon nanotubes have great research interest (Lihuan *et al.,* 2007). The single walled carbon nanotubes (SWCNTs) or multi walled carbon nanotubes (MWCNTs) are being functionalized on the surface by attaching various reacting groups on it. Carbon Nanotube (CNT), as a new class of nano materials, has been drawn considerable attention owing to their unique structure, high chemical stability and high surface-to-volume ratio. The ability of CNT to promote the electron transfer reactions of important biomolecules, such as cytochrome C (Xiang *et al.,* 2008), β- nicotinamide adenine dinucleotide [NAD] (Musameh *et al.,* 2002), ascorbic acid (Wang et al., 2002) and $H_2O_2$ (Wang *et al.,* 2003). Carbon nanotubes modified electrodes have great promise for dehydrogenase and oxidase based amperometric biosensors (Laurdi *et al.,* 2001; Lim *et al.,* 2003; Wang *et al.,* 2005; Huang *et al.,* 2006).

There are many drawbacks in the diagnosis of rheumatic infections by above described conventional methods. To increase the sensitivity and selectivity of diagnosis more diagnostic technique has to be proposed. Yuyan *et al* (2009) had explained about graphene based biosensor which offers high sensitivity, selectivity and low cost for the diagnosis of DNA sequence. Graphene sensor has inexpensive platform for patient diagnosis (Yuyan *et al.,* 2002). Kai *et al.,* 2009 *et al* reported an electrochemical DNA sensor based on chemically reduced graphene oxide. Scientists at Princeton University showed that single-stranded DNA strongly interacts with graphene, a nanomaterial made of sheets of carbon atoms just a single atom thick (Ping *et al.,* 2010). They also found that graphene protects DNA from break down by enzymes similar to those found in the body fluids which makes more durable (Fig.3). Such type of biosensors can be developed for diagnosis of rheumatic heart disease. Presently, biosensor is not available for diagnosis of pharyngitis and rheumatic heart disease.

## CONCLUSION

Although the incidences of rheumatic heart disease has considerably decreased in developed countries, but it remains a challenge to physicians who work in developing countries. Among all the diagnostic methods, throat cultures method is still applied in the microbiological laboratory for the preliminary detection of bacterium in the clinical specimens. However, RADT is currently quite popular with clinicians because they can be processed from fresh throat swabs. The specificity of RADT has been reported to be high as 95%, but sensitivity is considerably less and in case the result of RADT is negative, throat culturing is required. Because of all these limitations of diagnostic methods, a new technology with sensitive, reliable, rapid and economical for the diagnosis of RHD is required to save life of several people before damaging their heart valves. Biosensor and microarray based technology has provided a new pace in the area of molecular diagnostics.

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**Table 1:** Direct and indirect effect of environmental and health related factors on rheumatic fever and rheumatic heart disease (WHO technical Report Series, 2001)

| Determinants | Effects | Impact on RHD |
|---|---|---|
| Socioeconomic and environmental factors (Poverty, under nutrition, overcrowding, poor housing) | Rapid spread of streptococcal strains. | Higher incidence of acute streptococcal pharyngitis and suppurative complications, higher incidence of acute RF, higher rates of recurrent attacks. |
| Health-related factors: (Shortage of resources for health care, inadequate expertise of health care providers, low level awareness). | Inadequate diagnosis and treatment of streptococcus pharyngitis, misdiagnosis or late diagnosis of acute RF. | Higher incidence of acute RF and its recurrence. |

**Table 2:** Jones Criteria (1992)

| Major Criteria | Minor Criteria |
|---|---|
| Carditis | Fever |
| Polyarthritis | Arthralgia |
| Erythema marginatum | Elevated acute phase reactants (ESR, CRP) |
| Chorea | Prolonged PR intervals in ECG |
| Subcutaneous Nodules | |

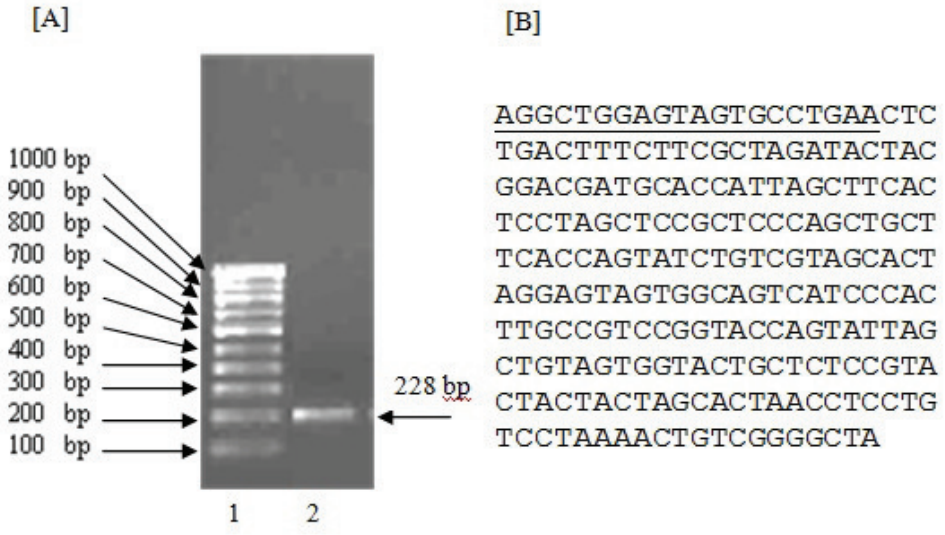


**Fig.1.** [A] Agarose gel electrophoresis (2.0%) of PCR product.Lane1: DNA Ladder 100bp; Lane2: purified PCR product (228 bp). [B] Sequence of PCR product 228 bp (Kumar *et al.,* 2011)

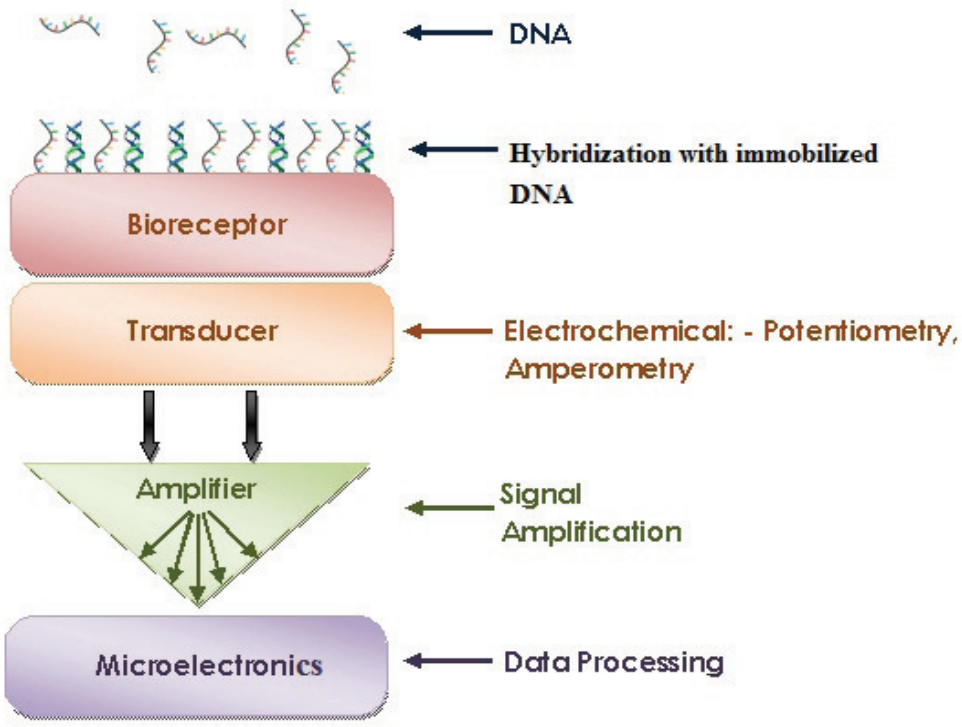

**Fig 2.** Schemematic representation of function of biosensors. DNA immobilized on the surface of electrode. Biological signals are transformed into electrical by transducer. Signals are amplified and readable by microelectronic devices.

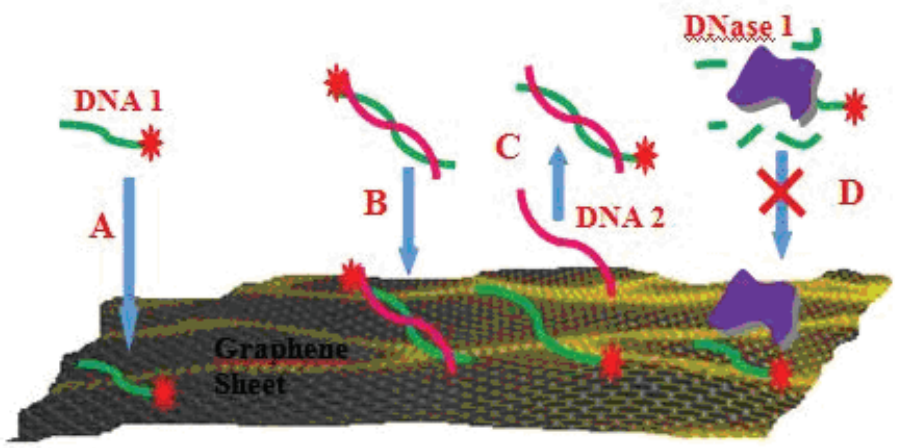


**Fig 3:** Interaction of fluorescent-tagged DNA with functionalized graphene. Both single-stranded DNA (A) and double-stranded DNA (B) are adsorbed onto a graphene surface, but the interaction is stronger with ssDNA, causing the stronger fluorescence on the ssDNA. C) A complimentary DNA when reaches to the adsorbed ssDNA at graphene surface causing detachment from the surface. D) DNA adsorbed onto graphene is protected from being broken down by DNase (Ping et al., 2010).

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