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Special Issue: Manual guidance of veterinary clinical practice and laboratory

Abdisa Tagesu*

Jimma University, School of Veterinary Medicine, Jimma, Oromia, Ethiopia

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***Corresponding author:** Abdisa Tagesu, Jimma University, School of Veterinary Medicine, Jimma, Oromia, Ethiopia, Tel: +251933681407, E-mail: abdisatagesu@gmail.com

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Research Article

Veterinary molecular laboratory examination

Molecular biological techniques are the best dimensions to diagnosis the disease process and its causative agents. The molecular biology technique is performed with sophisticated instruments or equipment's required specialized trained personnel and undertaken in routine diagnosistic laboratories. The importance of using molecular biology techniques is to diagnosis many bacterial and viral diseases genomes by giving the standard for protein resolution, nucleic acid resolution, and blotting techniques. The following techniques are used in veterinary molecular biology laboratory

Polyacrylamide gel electrophoresis

Polyacrylamide gelsare chemically cross-linked gels formed by the polymerization of acrylamide with a cross-linking agent, usually N, N'-methylenebisacrylamide and the high molecular weight complex compounds are allowed to pass through gel matrix of the polymer of acrylamide by applying electric current (Figure 1). This method has high resolution capacity which can be further enhanced by addition of sodium dodecyl sulfate (SDS) which allows the resolution on the basis of molecular weight of the compound [1].

The Required Reagents: In the polyacrylamide gel electrophoresis, one requires 0.2 M sodium phosphate buffer (pH 7.2), acrylamide-bisacrylamide ratio (22.2:0.6), ammonium persulfate (15 mg/ml distilled water), TEMED (N, N, N', N'-Tetra methylethylene diamine), bromopheno1 blue (50 mg/100 ml distilled water) and staining solution, 50% Glycerol, 1X TBE , 0.5% xylene cyanol, Ethidium bromide, 10 microgram/ ml.

Procedure of Gel Electrophoresis [2]:

- Prepare an appropriate gel volume that is more than sufficient to fill the chamber formed between the gel plates. Mix gentle stirring the 44 ml water, 5% gel mix and 50 ml of 10% acrylamide solution for 100ml chamber.
- Before adding gel mix into chamber, add 1 ml of 10% ammonium persulfate and 75 micro litre of temed.
- Mix gently using a 50 ml syringe without a needle and dispense mixture into the chamber using the syringe and fill the space entirely. \Promptly insert slot former into the top of the chamber and clamp tightly in place and allow gel to polymerize 15 min to 1 hr.
- After polymerization, remove bottom spacer and slot former, and mount gel in electrophoresis apparatus and fill upper and lower tanks with 1x TBE buffer, submerging upper slots.
- Remove any trapped bubbles from the space under the gel in the lower tank.
- The water layer is then removed and the tubes are placed in gel apparatus. In 40 ml solution of protein (sample, which may be bacteria/ virus/ rickettsia/chlamydia/ myoplasma in purfied form), add 10 ml of tracking dye (bromophenol blue 0.05%) by using micropipette to each gel. Also use positive control to compare the results.

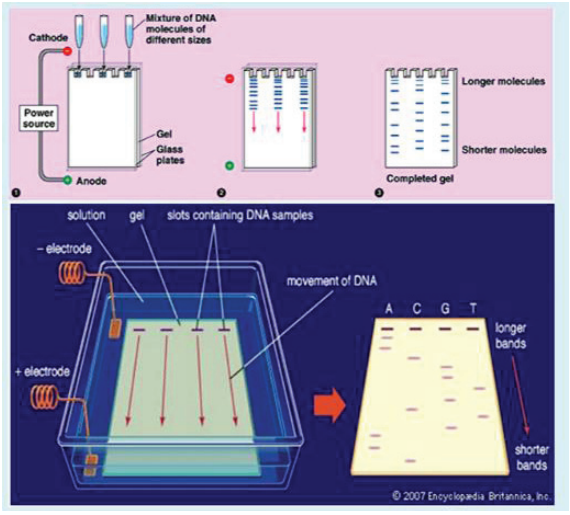

Figure 1: Structure od Gel Electtophoresis.

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- Run the gel by applying a voltage of 2- 10 V per centimeter of gel and run the gel long enough to resolve the sample fragments of interest. As a rough guide, the bromophenol blue dye front comigrates with DNA fragments of about 50 bp, whereas the xylene cyanol dye front runs with 400 bp fragment in a 5% gel.
- Turn off power supply and remove the gel from apparatus.
- Separate the two plates with gel and adhering to one of them and the resolved DNA can now be detected by autoradiography if radiolabeled or ethidium bromide staining.
- Autoradiography may be performed on the wet gel by covering it with clear plastic wrap and applying it to XAR-5 film in a cassette.
- Gel can be transferred to whatman 3MM paper and covered with clear plastic wrap. This gel can be autographed or dried on a gel dryer and the autoradiographed.
- Ethidium bromide staining can be performed by carefully layering 50-100 ml of an ethidium bromide solution, 10 microgram/ ml, onto the gel while it is still adhering to one of the plates. Allow ethidium bromide solution to diffuse into gel and stain DNA for 15 minute.
- Gently rinse excess stain from the gelsurface with water and cover the gel with clear plastic wrap and visualize DNA by UV transillumination [plastic wrap directly against UV source, because glass absorbs UV light.
- Gel may now be photographed and identified fragments can now be cut from gel with a new razor blade and recovered by electroelution.

Nucleic acid hybridization

The nucleic acid hybridization is the process wherein two DNA or RNA single chains from different biological sources, make the double catenary configuration, based on nucleotide complementarity and of contingent sequence homology of the two sources, resulting DNA-DNA, RNA-RNA or DNA-RNA hybrids (Figure 2) [3]. After electrophoresis, the cells are plated on an agar solidified medium and blotted with a disc of nitrocellulose paper. The paper later treated to lyse cell and dissociate DNA to be hybridized with the probe and finally to be auto radiographed [4]. For this the nitrocellulose paper is applied on both sides of the gels to get duplicate blots of the gel.

The nucleic acid used to detect the presence of specific nucleic acid in the immobilized sample is radioactively labeled is known as proble. If complementarity exists between the probe and the immobilized nucleic acid, hybridization will occur and this can be measured by a scintillation counting or authoradiography and it is known as dot-blot hybridization [5,6]. When hybridization takes place on a solid carrier is named blotting and is divided in 3 categories: Southern

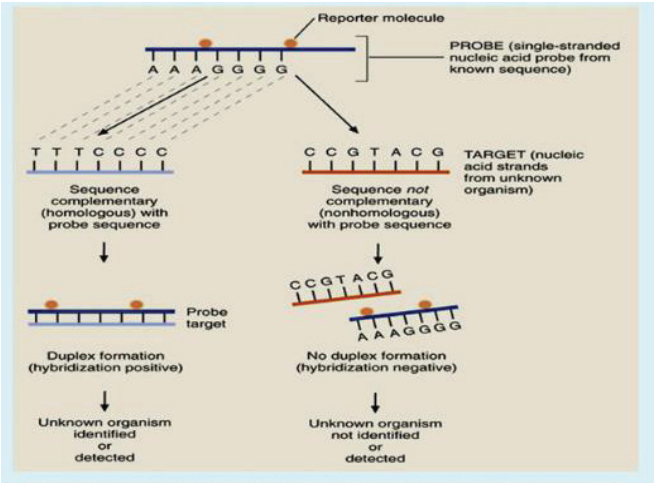

Figure 2: Principles of nucleic acid hybridization. Identification of an unknown ogganisam is established by postive hybridization (duplex formation) between a nucleic acid strand from the known sequence (probe) and a target uncleic acid strand from the organism to be identified. Failure to hybridize lack of homology between the probe and target uncleic acid.
(http://basicmedicalikey.com).

blotting whereby DNA molecules are identified using DNA or RNA probes; Northern blotting whereby RNA molecules are identified using RNA or DNA probes; Western blotting whereby protein sequences are identified using specific antibodies [3]. After overnight blotting, the nitrocellulose membranes are slightly rinsed in distilled water and heated at 80°C for 2 hours.

The membranes are then key at room temperature till hybridization. For hybridization, specific probes are used in order to make a confirmatory diagnosis. The probes are purified, characterized, nucleic acid sequence, which can be specific for a given species of organisms [7,8]. The hybridization probe is a fragment of DNA or RNA of variable length (usually 100–1000 bases long) which can be radioactively labeled. It can then be used in DNA or RNA samples to detect the presence of nucleotide sequences (the DNA target) that are complementary to the sequence in the probe. The probe there by hybridizes to single-stranded nucleic acid (DNA or RNA) whose base sequence allows probe target base pairing due to complementarity between the probe and target (Figure 3,4) [8].

There are two ways how to synthesis probes like nick translation and random primers.

Probe synthesis

Probes canbe synthesed by using randomized primers and nick translation.

Probe synthesis by "nick-translation"

The DNA molecule double stranded is subject to DN-ase I action that is a nonspecific site endonuclease and that takes to pieces randomly, phosphodiesteric bundles, in the presence of Mg^{2+} ions, generating single chain breaks "nicks"; as a result of exonuclease 5-3'action of a DNA polymerase I from *E. coli* nicks are enlarged simultaneously with exonuclease 5-3'action of DNA polymerase I, this shows its 5'-3'polymerizationactivity using marked dNTPs and thus a translation of the single chain

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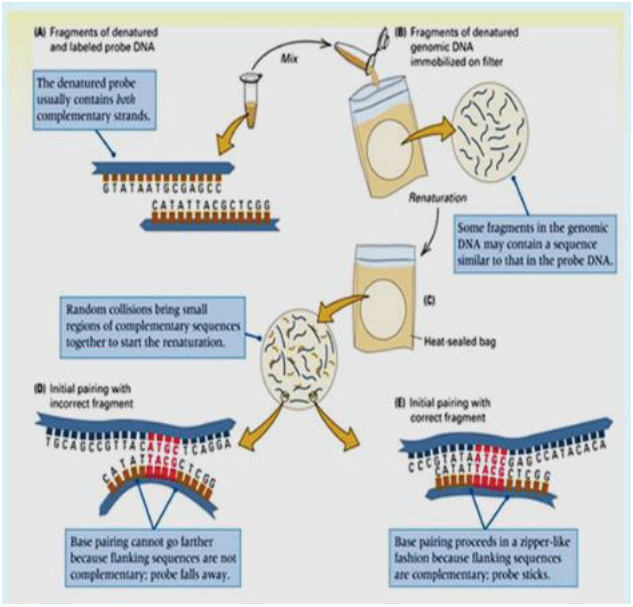


Figure 3: Nucleic acid hybridization process.

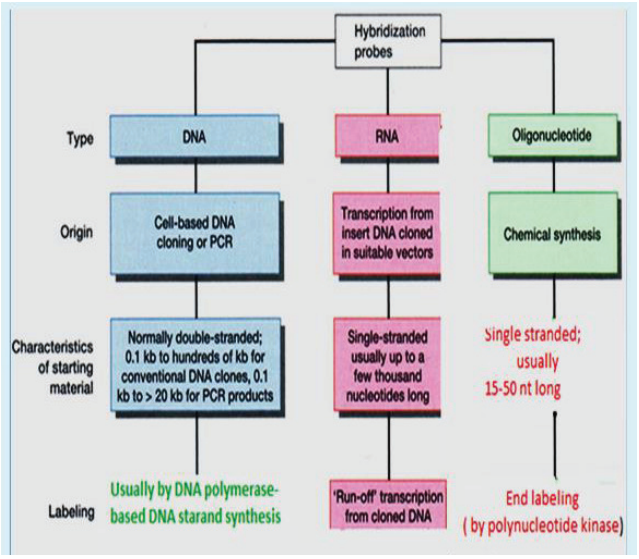


Figure 4: Hybridization of DNA and RNA.

break is carried out (nick-translation). DNA double staranded sequences thus obtained and marked uniformly are subject to thermal denaturation by breaking the hydrogen bridges and obtaining single chain probes.

Probe synthesis using randomized primers.

Randomized primers are heterogenous sequence oligonucleotides that can hybridize in many sites of the matrix chain. DNA is isolated from the salmon sperma or calfe timus and is acted over it with DN-ase I so as a high population of single chain DNA oligonucleotide sequences are obtained having dimensions of 6-12 nucleotides. Automaticoligonucleotide synthesizer having all the 4 types of dNTPs in the reaction medium. Thermic denaturation of the matrix and production of DNA single chains, Attaching of randomized primers in various sites of single chains (at random).

Using 3 types of simple nucleotides and one marked radioactively, in the persence of DNA polymerase I and Klenow-DNA pol I(it has no exonuclease 5'-3'activity), completion of holes among primers, by copying the matrix information, By heat denaturationradioactively marked probes are obtained.

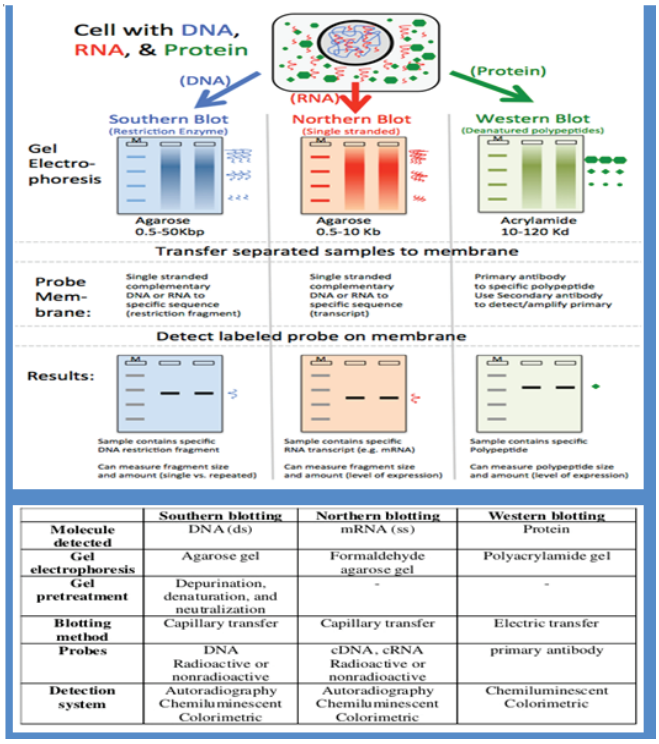
The probes are of two types they are detailed as below (Figure 5) [3].

Radio Labelled Probes: Mostly used radioactive labels are phosphorous 32 (^{32}P), sulphur 35 (^{35}S) and tritium (^{3}H) which are detected by process of autoradiography. These probes are used for identification and detection of the probes after hybridization [9].

Biotin Labelled Probes: Biotin labeled probes are non-radioactive like enzyme reactions (peroxidase, alkaline phosphate) and luminescence (Adamantyl Phosphate derives,Lumi-Phos). Instead of radioactive material, the probe is labelled with biotin which is detected by avidin-peroxidase conjugate by using an enzyme substrate like diaminobenzidine tetrahydrochloride. The colour developed by the substrate at tlie reaction site is visible by naked eye; and the blotted nitrocellulose sheets can be stored for long period. Blotting is the technique in which nucleic acids or proteins are immobilized onto a solid support, generally nylon or nitrocellulose membranes [10].

Types of blotting

The electrophoresis of nucleic acid and protein blotting and its detection by using probes is classified into three types[11, 12] (Figure 6).



	Southern blotting	Northern blotting	Western blotting
Molecule detected	DNA (ds)	mRNA (ss)	Protein
Gel electrophoresis	Agarose gel	Formaldehyde agarose gel	Polyacrylamide gel
Gel pretreatment	Depurination, denaturation, and neutralization	-	-
Blotting method	Capillary transfer	Capillary transfer	Electric transfer
Probes	DNA Radioactive or nonradioactive	cDNA, cRNA Radioactive or nonradioactive	primary antibody
Detection system	Autoradiography Chemiluminescent Colorimetric	Autoradiography Chemiluminescent Colorimetric	Chemiluminescent Colorimetric

Figure 5: Comparison of southem, northem and westem blotting techniques.

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Southern blotting: It is a method used for detection of a specific DNA sequence in DNA samples. DNA is separated by electrophoresis, then trnsffered to nitrocellulose paper and denature into single strands that can be hybridized with a specific probe[13] (Figure 7).

Procedure [7,14,15]:

* High molecular weight DNA strands are cut into smaller fragments by restriction endonucleases.
* The DNA fragments are separated by size by agarose gel electrophoresis and then transferred to a nitrocellulose membrane which is placed on the top of the gel.
* In Sourthen blotting, before transfer, DNA is usually denatured with alkali for denaturation of the double stranded DNA.
* The denaturation in an alkaline environment may improve binding of the negatively charged DNA to a positively charged membrane, separating it into single DNA strands for later hybridization to the probe, and destroys any residual RNA that may still be present in the DNA.
* After transfer of the DNA fragments to the nitrocellulose membrane which is done by capillary action or may be by electrotransfer, vacuum transfer or centrifugation, the membrane is then baked in a vacuum or regular oven at 80 °C for 2 hours to permanently attach the transferred DNA to the membrane. The membrane is then exposed to a hybridization probe (a single DNA fragment with a specific sequence whose presence in the target DNA is to be determined).
* The probe DNA is labelled so that it can be detected, usually by incorporating radioactivity or tagging the molecule with a fluorescent or chromogenic dye. After hybridization, excess probe is washed from the membrane, and the pattern of hybridization is visualized on X-ray film by autoradiography in the case of a radioactive or fluorescent probe or by development of color on the membrane if a chromogenic detection method is used.

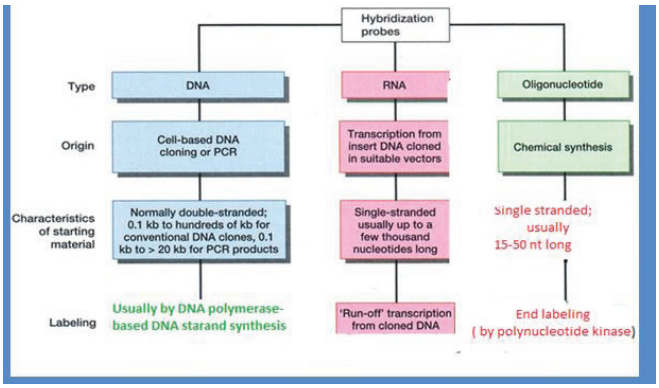

Figure 6: Hybridization of DNA and RNA probes.

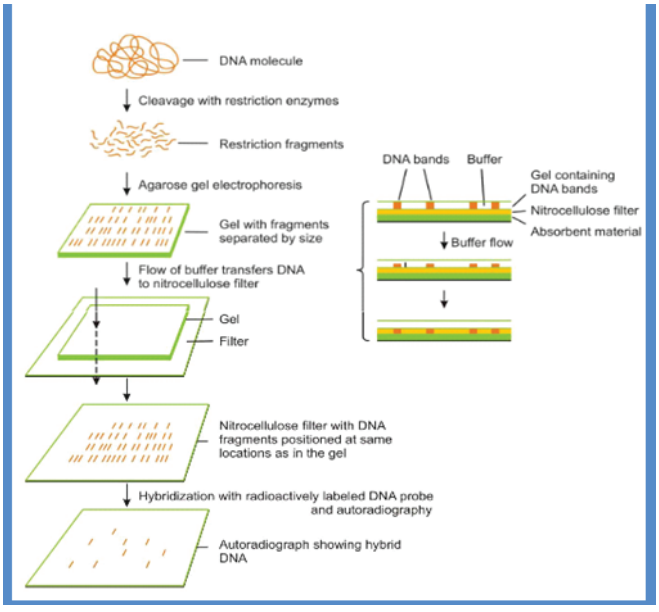

Figure 7: Procedure of southern blotting in sepration of DNA.

Northern blotting

The north blot is a techniques used in molecular biology research to study gene expression by detection of RNA (isolated mRNA) in a sample [16]. When the RNA is electrophoresed and blotted on nitrocellulose paper and is detected by nucleic acid probe. The northern blot technique is used to study gene expression by detection of RNA (or isolatedmRNA) in a sample. It is used to identification and qualification of RNA expressed in cells. The RNA population is separated by size during gel electrophoresis followed by transfer of the RNA onto a membrane (nitrocellulose or nylon) [17].

Procedure [7,18]:

The procedure includes the following steps RNA isolation, Probe generation, denaruring, hybridization and washing and detection (Figure 8):

- The nucleic acid molecules (RNA samples) are separated by agarose gel electrophoresis and then transferred to a nitrocellulose membrane but for RNA in Northern blotting, alkali denaturation is not necessary and would in any case hydrolyze the molecules.
- A nylon membrane with a positive charge is the most effective for use in northern blotting since the negatively charged nucleic acids have a high affinity for them.
- The transfer buffer used for the blotting usually contains formamide because it lowers the annealing temperature of the probe-RNA interaction, thus preventing RNA degradation by high temperatures. Once the RNA has been transferred to the membrane, it is immobilized through covalent linkage to the membrane by UV light or heat.
- After a probe has been labeled, it is hybridized to the RNA on the membrane. Experimental conditions that

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can affect the efficiency and specificity of hybridization include ionic strength, viscosity, duplex length, mismatched base pairs, and base composition.

- The membrane is washed to ensure that the probe has bound specifically and to avoid background signals from arising. The hybrid signals are then detected by X-ray film.

Western blotting

Western blotting does not involve nucleic acid hybridization. The western blot (alternatively, immunoblot) is used to detect specific proteins in a given sample of tissue homogenate or extract. Proteins are separated on a gel, transffered to amemebrane and detected by antibodies. When protein is electrophoresed and is detected by using monoclonal or polyclonal antibodies and enzyme conjugates and substrate and by producing a coloured visible reaction; it is known as western blotting or immunoblotting [19].

Procedure (Figure 9):

The principle of the Western blotting is based around a few broad steps [20]:

- The extraction of cellular proteins from a complex mixture of intracellular and extracellular proteins (from tissue, cells, etc.).
- Quantification of protein concentration and electrophoretic separation of proteins within a gelmatrix; transfer to a membrane with a high affinity for proteins;
- Blocking" the membrane to reduce non-specific binding;

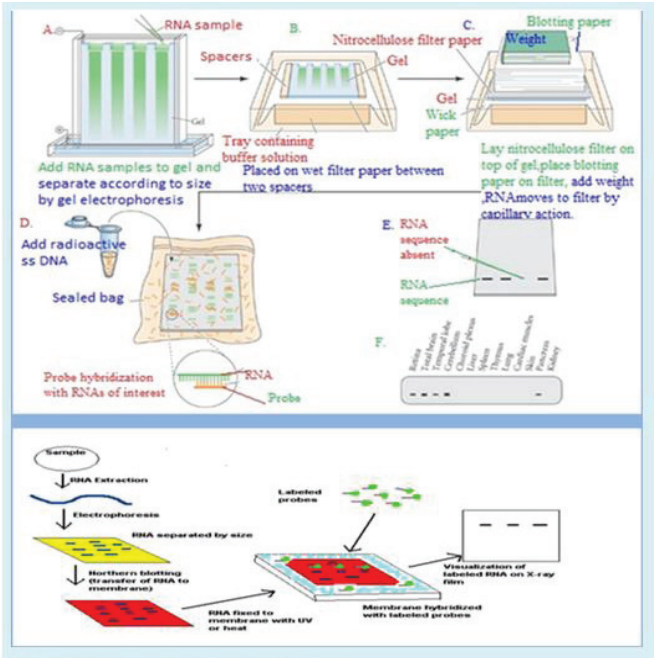

Figure 8: Northern blotting for RNA identification.

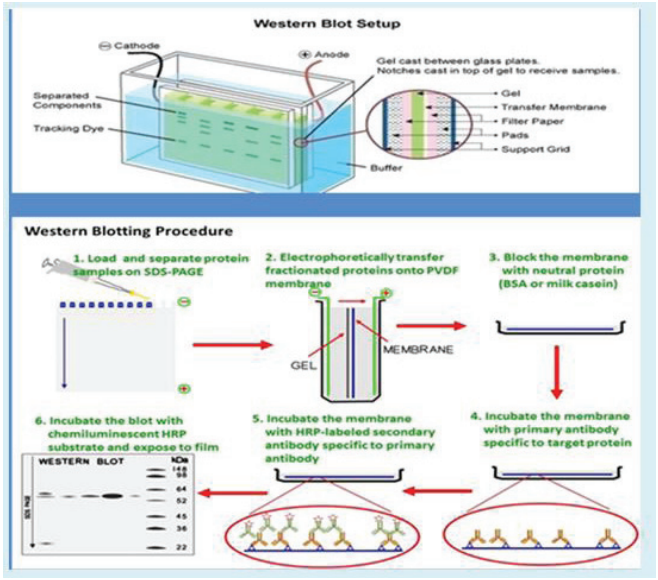

Figure 9: Procedure and technique of western blotting.

- Antigen detection by antibodies specific for the protein(s) of interest;
- Incubation with a secondary antibody linked to a label (e.g., chemiluminescent or fluorescent);
- Development and detection of the signal, which is theoretically proportional to the degree of antigen/ antibody binding (Figure 10).
- Quantification of the resulting bands using densitometry

Polymerase Chain Reaction (PCR)

The PCR is a widely used technique in molecular biology because of its high sensitivity, specificity and user-friendly nature. In 1971, the idea by Kjell Kleppe [21] was described to replicate short DNA fragments by nucleotide primers *in vitro*, but the invention of the complete assay is credited to Kary Mullis [22]. By the development of related disciplines (enzymology, oligosynthesis, electromagnetism, etc.), there is a large variety of PCR technologies available nowadays [23]. At the present time, the PCR is based on a thermostable DNA polymerase, which amplifies a specific region of the target DNA initiated by short, 15-30 bp long oligonucleotides (primers), following the principle of Watson-Crick base pairing.

PCR is a highly sensitivity procedure for detection of infectious agents in host tissues and vectors. Pcr can target and amplfy a gene sequence that has become integrated into the DNA of infected host cells. However, PCR do not differentiate viable and nonviable organisms or incomplete pieces of genomic DNA.PCR may use to diagnosis chronic persistence infection like retroviruses bovine leukoaemia virus, caprine arthritis/ encephalitis virus [24].

The material which required in processing of PCR are listed below [25,26]:

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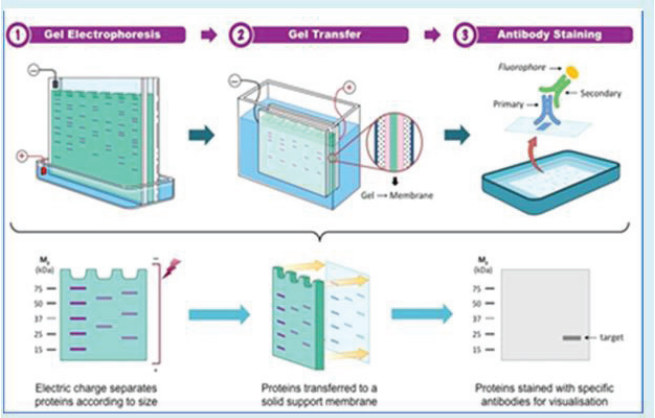

Figure 10: Gelelectrophoresis procedure in identification of antibodies.

- 1.5 ml microcentrifuge tubes
- 0.2 ml PCR tubes (16 tubes)
- Microcentrifuge tube rack
- Empty tip boxes for holding PCR tubes
- The streak plates of your starch degraders
- Inoculating needle
- Micropipettors and tips
- Purified soil DNA
- Nuclease-free H2O
- 10X PCR buffer
- 25 mm MgCl2
- Deoxynucleotides (1.25 mm dNTPs each)
- Forward primer (designated 11F)
- Reverse primer (designated 1492R)
- *Taq* polymerase (*Taq* polym-erase)
- Positive control

The PCR reaction requires the following components [27]:

DNA Template: The double stranded DNA (dsDNA) of interest, separated from the sample.

DNA polymerase: Usually a thermostable Taq polymerase that does not rapidly denature at high temperatures (98°) and can function at a temperature optimum of about 70°C.

Oligonucleotide primers: Short pieces of single stranded DNA (often 20-30 base pairs) which are complementary to the 3' ends of the sense and anti-sense strands of the target sequence.

Deoxynucleotide triphosphates: Single units of the bases A, T, G, and C (dATP, dTTP, dGTP, dCTP) provide the energy for polymerization and the building blocks for DNA synthesis.

Buffer system: Includes magnesium and potassium to provide the optimal conditions for DNA denaturation and renaturation; also important for polymerase activity, stability and fidelity.

All the PCR components are mixed together and are taken through series of 3 major cyclic reactions conducted in an automated, self-contained thermocycler machine.

Denaturation: This step involves heating the reaction mixture to 94°C for 15-30 seconds. During this, the double stranded DNA is denatured to single strands due to breakage in weak hydrogen bonds.

Annealing: The reaction temperature is rapidly lowered to 54-60°C for 20-40 seconds. This allows the primers to bind (anneal) to their complementary sequence in the template DNA.

Elongation: Also known at extension, this step usually occurs at 72-80°C (most commonly 72°C) to raise the reaction temperatures so *Taq* polymerase extends the primers, synthesizing new strands of DNA. In this step, the polymerase enzyme sequentially adds bases to the 3′ each primer, extending the DNA sequence in the 5′ to 3′ direction. Under optimal conditions, DNA polymerase will add about 1,000 bp(base pair) /minute.

Final elongation: This single step is optional, but is performed at a temperature of 70–74 °C (158–165 °F) (the temperature range required for optimal activity of most polymerases used in PCR) for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully elongated.

Final hold: The final step cools the reaction chamber to 4–15 °C (39–59 °F) for an indefinite time, and may be employed for short-term storage of the PCR products.

The Requirements in processing of PCR in veterinary molecular laboratory like: [26,28-33]:

Thermostable DNA polymerase: The use of thermostable DNA polymerases in I'CR has made it simple because single addition at the beginning is sufficient for the entire amplification process without any futher requirement. These enzymes are obtained from thermopilic bacteria that have capacity to replicate at higher temperatures. Such enzymes are also produced in E. *coli* through recombinant DNA technology and at present both natural and recombinant enzymes are commercially available. These include Taq polymerase, AmpliTaq, Vent, pfu DNA polymerase, TthDNA polymerase, UITma etc.

Deoxyribonucleic acid Triphosphates: It is used in 200 pM concentration which is enough to synthesize12.5 pg DNA. Each batch of dNTP should be checked for pH andpH 7.0 should be adjusted using lN NaOH.

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Primer: Primer is oligonucleotide sequence with 17-30 nucleotides having 50% GC content 5' end primer is used for PCR product modification like addition of restriction site but 3' end should be intact without any inter or intra-primer complementarity which may lead to primer dimmer formation in PCR.

PCR primers are specific short string of ss DNA known as oligomers [Various kinds of primers are as under:

Random primers: *These are random hexamers of four nucleotides A, T, G and C and are used for c DNA synthesis. Universal primers-Primer sequences flanking the cloning sites of plasmids are termed as universal primers.*

Degenerate primers:*Every possible combination of nucleotides that code for a given amino acid sequence is called degenerate primers.*

Specific primers:Primers designed to amplify a specific target DNA of defined length.

News fed Primers:Primers used to amplify a segment interval to the previously amplified PCR product.

Buffers and Magnesium Chloride: The most commonly used buffers (lox) include 100 mm Tris hydrochloride, 500 mM potassium chloride and 15 mM magnesium chloride. It also contains 0.1% (w/v) gelatin and has pH 8.5 at room temperature. Magnesium is a required cofactor for thermostable DNA polymerases, and magnesium concentration is a crucial factor that can affect amplification success. It is used with taq or Ampli Taq DNA polymerase. However, buffer should be used as per the recommendatlom of supplies and it may vary with DNA polymerase. Magnesium chloride is very critical in PCR and is responsible for formations of soluble complex with d NTPs which is essential for incorporation. It also stimulates polymerase activity, increases Tm of ds DNA and promotes primer annealing.

Low concentration of magnesium chloride results in low yield while excess concentration may lead to accumulation of non-specific products. The EDTA used for dissolving DNA should not have more than 0.1 mM concentration otherwise it will affect magnesium ion concentration.

Template DNA: Sample is prepared by lysing the cells through boiling in hypotonic solution. Care should be taken to avoid contamination of template DNA with PCR inhibitors which include heparin, protease-K porphyrin, ionic and nonionic detergents and phenol.

Procedure of PCR how to perform in veterinary disease diagnosis (Figure 11) [34,35]:

- Prepare the reaction mixture in PCR tube as following
  - ✓ Nuclease-free water - 59.0 µ1
  - ✓ 10 x PCR buffer - 5.0 µ1
  - ✓ dNTPs (2mM each) -1.0 µ1
  - ✓ Primer I(100 p moles) - 1.0 µ1
  - ✓ Primer I1 (100 p moles) - 1.0 µ1
  - ✓ Template DNA - 2.0 µ1
- Mix well and centrifuge at 5000 rpm for 15 sec and add 1 µ1 enzyme Taq polymerase (2.54/Ml).
- Mix well and again centrifuge at 5000 rpm for 15 sec.
- Heat the PCR mixture at 94°C for 4 min for denaturation.
- Again heat the PCR mixture at 94°C for 1 min.
- Incubate the mixture at 55°C for 1 min.
- Incubate the PCR mixture at 72°Cfor 3 min.
- Repeat the process from step 5 to 7 for 54 cycles, however, the number of cycles depends on denaturation temperature, duration and amount of template DNA. At higher denaturation temperature, enzyme activity is lost and more number of cycles would not help. If the template concentration is low, the number of cycles can be increased.
- After 54 cycles, incubate the mixture at 72°C for 5 min. This is the final ex tension step to ensure all PCR products are blunt ended.
- Cool the product in ice. It should be immediately chilled and should not be left at room temperature as the residual enzyme activity may lead to formation of non-specific product.
- For amplification of RNA by PCR, complementary DNA (cDNA) is synthesized first using reverse transcriptase and then cDNA is amplified in PCR as given above

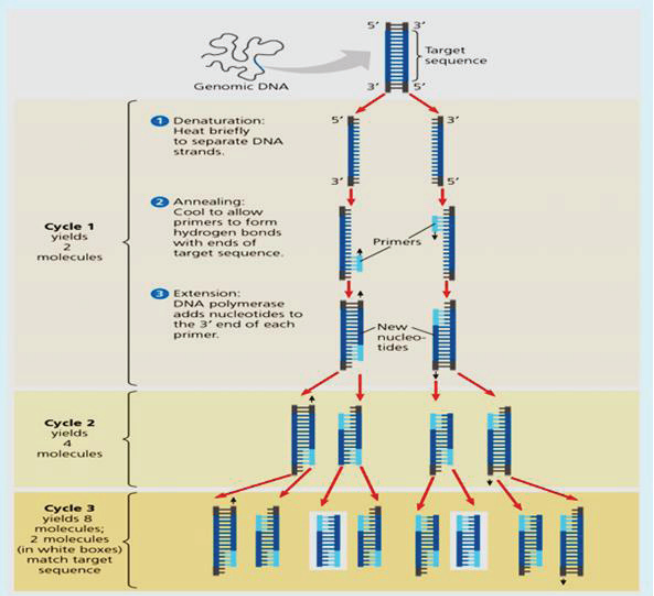

Figure 11: The procedure of PCR.

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➢ Confirmation of PCR product is the ultimate result of PCR standardization.

The PCR amplified product is confirmed through either of the following methods [36-39]:

- Agarosegel electrophoresis: It can confirm the size of product when run in 1% agarose with molecular weight markers and dye.
- Restriction enzyme analysis: The product can be fragmented using resitriction enzyme and then run on agarose gel for expected fragments.
- Nested primer PCR: In this, amplify a region of PCR product using nested primers that flank the region internal to the product and confirm the specific PCR product.
- Nucleotide sequencing: Sequence the PCR product for confirmation.
- Southern blotting: Transfer the PCR product on nitrocellulose after electrophoresis and carryout hybridization using DNA probe.

Interpretation of PCR

Positive PCR: When comparing with other pathogen isolation PCR usually generates more positive results because it requires much less pathogen.Positive PCR canbe obtained in cases in which viral culture and isolation test results are negative due to PCR detects pathogen DNA rather than active pathogen reproduction and also it can identify asymptomatic animals that harbor nonviable organisms[40].Animals that harbor a viable pathogen can be identified if the intial screening PCR is followed by culture or viral isolation. False-positive PCR results can occur through the undetected inclusion of contaminating DNA within the test reaction[41]:

✓ Excessive PCR cycling resulting in amplification of DNA similar to the target DNA

✓ low specificity of the PCR primers (primers that amplify similar nonpathogen DNA)

✓ Carryover contamination in post-PCR analysis using conventional PCR techniques. Contamination can be monitored through inclusion of negative control reactions in which the test protocol is identical, but target DNA is not included.

The development of a new PCR test should include primer testing using target DNA with similar sequences (e.g., primers for equine herpesvirus should be tested against those for equine herpesvirus 4) to ensure that the primers have good specificity to the target DNA [42]. The exponential amplification of pathogen genetic material using PCR generally leads to an increase in positive results compared with most other conventional methods. In addition to the basic PCR technique, other advances in PCR technology (nested PCR, real-time PCR) have been used to increase the specificity and sensitivity of PCR-based diagnostic testing [43].

Negative PCR: Negative PCR results from infected animals which occur when samples from infected animals do not contain target DNA or RNA. Negative PCR results can be isolated from cerebrospinal fluid samples that lack of bacterial infection or from serum of infected animals that have passed short lived viremic phase of infection with encephalitis virus [44]. False negative PCR results can occur when samples contain appropriate target DNA, no DNA are amplified but pathogen DNA are present due to handling or laboratory errors(poor DNA extraction from samples, DNA degradation, poor primer performance, poor reaction optimization, transcription errors and presence of contaminating inhibitory substances in the samples [45-47].

Polymerase chain reaction- enzyme linked immunosorbent assay technique

Polymerase chain reaction-enzyme linked immunosorbent assay (PCR-ELISA) is an immunodetection method that can quantify PCR product directly after immobilization of biotinylated DNA on a microplate. It is used to detect bacteria, fungi, viruses and other pathogenic organisms [48,49]. It can detect nucleic acid of microorganism instead of protein (figure 12), it is more sensitive method compared to conventional PCR method, with shorter analytical time and lower detection limit [48]. PCR-ELISA is a more accurate diagnostic test that other molecular and serological tests [50].

PCR-ELISA methods can be undergone in three steps [48,51] and also indicated in figure 12:

* Amplification: The gene of microorganism is amplified through PCR in the presence of digoxigenin-11-dUTP (DIG-dUTP). DIG-labelled PCR products will then bind to specific oligonucleotide probes, labelled with biotin at their 5′ end.

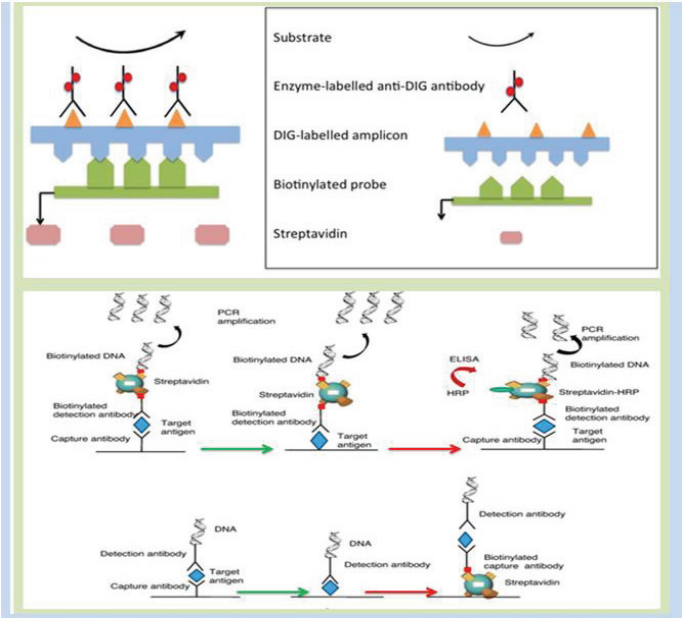

Figure 12: The methods of immuno-PCR or PCR-ELISA technique in detection of microbial genome instead of the protein (antigen).

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* Immobilization: Immobilization of interested gene of isolated gene into microplate. Presence of streptavidin coated on plates and biotin on the 5' end of the formed hybrid can viable the procedure. Affinity of avidin-biotin interaction forms avidin-bitin complexes; this allows only PCR products to bind with specific gene interest to microplate. Other uninterested product could be washed out.

* Detection: the formed complexes can't be detected by naked eyes, therefore it can be detected by using an anti-DIG-peroxidase conjugate through substrate 2,2' azino-di-3-ethylbenzythiazoline sulfonate. Form this conjugation blue-green color reaction will be developed, this is visible and also measure by spectrophotometer.

* Fluorescein probe can also detect the complexes formed in PCR-ELISA detection, using antifluorescein antibodies conjugated to horseradish peroxidase to detect the hybrized fluorescein labeled oligonucleotide probe.

PCR-ELISA technique is used to detect parasite, fungi and bacteria from sample of diseased animals [52-55].

* The first step for detection of microorganism is that collection of sample form diseased animals, fecal sample, blood and etc.

* Then DNA of the agents can be isolated from sample

* Identification of primer used for amplification: the primer used for amplification for *Trypanosma spps* are listed as following :

* Prime for *Trypanosome vivax* : ILO1264 (CAG CTC GGC GAA GGC CAC TTG GCT GGG) and biotinylated ILO1265 (biotin-TCG CTA CCA CAG TCG CAA TCG TCG TCT CAA GG), the fluorescein-tagged oligonucleotide for hybridizing with the PCR products obtained is designated violo-3, and has the sequence: (FL-CA GAG CAG TCT CGG CGC GCC CCA TGT TC-FL). The biotinylated oligonucleotide designated violo-4 (biotin-CT GGA GGT GAA CAT GGG GCG CGC CGA GAC TGC TCT G) is used as a positive control when detecting by ELISA the PCR product of *T. vivax* DNA amplification [55].

* *Primer for T. brucei:* the pair of primers designated bruolo-1 (biotin-AA GAA CCA TTT ATT AGC TTT GTT GC) and bruolo-2 (CGA ATG AAT ATT AAA CAA TGC GCA G). The fluorecein-tagged oligonucleotide primer designated bruolo-3(5-FL-CAA TGT GTG CAA TAT TAA TTA CAA GTG TG-FL-3) is hybridized with the PCR products obtained. The primer, bruolo-4 (biotin-TAT TTA ATG TTG CAC ACT TGT AAT TAA TAT TGC AC ACA TT), is used as a positive control when detecting by ELISA the PCR products of T. brucei DNA amplification, because it is complementary to the fluorescein-tagged oligonucleotide primer [55].

* The following steps are undergone to detect the PCR product by ELISA:

✓ The ELISA is performed in microtitre plates coated with streptavidin diluted in carbonate coating buffer to detect the PCR product.

✓ The plates were shaken briefly after addition of the coating buffer, incubated at room temperature for 2 h, and then stored at 4 oC for up to 1 week after removal of the buffer.

✓ Wells of the microtitre plates is washed six times with Tris-buffered saline

✓ The PCR products is analyzed by ELISA diluted 1:10 with hybridization buffer and 100 ml of each sample is dispensed into each well and incubated at 37oC for 1hr to facilitate the binding of the biotinylated PCR products to the streptavidin coating in the wells of the plate.

✓ The PCR product binded to streptavidin is denatured by adding NaOH in each well and incubating for 2 min at room temperature.

✓ The unbound PCR product is removed by washing the plates six times with TBS/Tween-20.

✓ Fluorescein-labeled oligonucleotide probe (at 1 ng/ml) in hybridization buffer is added into each well and the plate incubated at 55 oC for 2 hr.

✓ The fluorescein-labeled probe is revealed by introducing into each well of anti-fluorescein antibody conjugated to alkaline phosphotase, diluted in TBS/1% BSA.

✓ The reaction proceeded at 37 °C for 30 min.

✓ An amplifier (alcohol dehydrogenase plus diapherase) is added at well and the assays transferred to 37oC for 6 min.

✓ The action is terminated within a 6 min interval, by the addition of sodium chloride into each well.

✓ The optical densities of the reactions in each of the wells can be determined using an ELISA microtitre plate reader

✓ From interpretation, the conjugation of PCR product and substrate of ELISA produces blue green color reaction which can be measure by spectrophotometer.

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