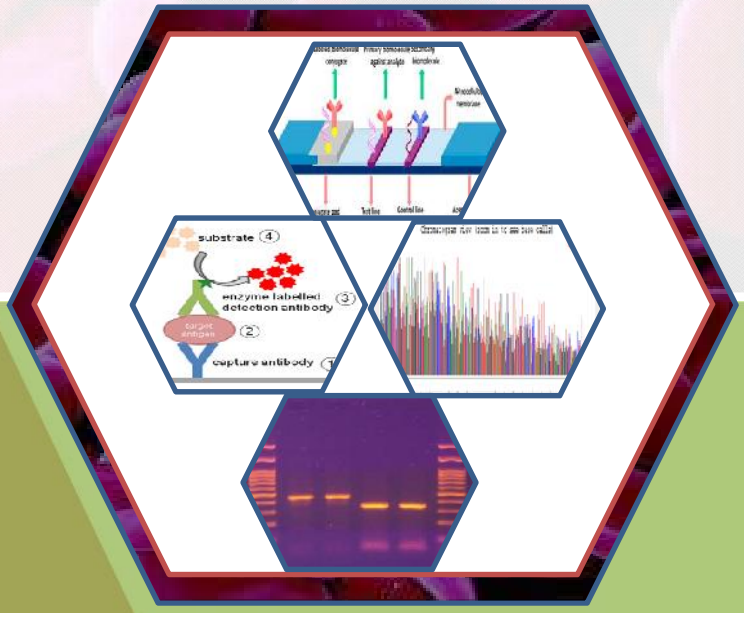
Training Manual
On
An update of molecular and advanced approaches for diagnosis of parasitic diseases of animals
(2-11th January, 2019)



Organized by:

ICAR - National Institute of Veterinary Epidemiology and Disease Informatics, Ramagondanahalli, Yelahanka, Bengaluru

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(2-11th January, 2019)

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Sponsored by

Indian Council of Agricultural Research

Organized by:

ICAR - National Institute of Veterinary Epidemiology and Disease Informatics, Ramagondanahalli, Yelahanka, Bengaluru

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We wish to express our sincere gratitude to Indian Council of Agricultural Research for selecting our training proposal and sponsoring the programme. We are grateful to the DDG (AS), DDG (HRD), ADG (HRD), ADG (AH), for their support, encouragement and best wishes.

We extend our heartfelt gratitude to our beloved Director (Acting) Dr. B. R. Shome for his guidance and constant encouragement. Our sincere thanks are due to the authorities of the institutes/ universities who granted deputation of the participants.

It is our pleasure to express our gratitude to all the external and internal faculty members of this training programme, for contributing their valuable time in preparation of write-up, delivering lecture, presentation and practical demonstration. Our sincere thanks are also due to all the committee members of this training programme for their generous help.

We offer our sincere thanks to all the scientific, technical, administrative, financial, supporting staffs, JRFs, contractual staffs who assist us to run the training programme smoothly.

Dr. P.P. Sengupta (Course Director)

Dr. M. Nagalingam (Course Co-ordinator)

Dr. S. S. Jacob (Course Co-ordinator)

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Dr. Joykrushna Jena
Deputy Director General
(Animal Science)

MESSAGE

I am immensely delighted to know that ICAR- National Institute of Veterinary Epidemiology and Disease Informatics (ICAR-NIVEDI), Bengaluru is organizing an ICAR- sponsored short course on 'An update on molecular and advanced approaches for diagnosis of parasitic diseases of animals' from 2nd to 11th January, 2019.

In developing countries, livestock contribute up to 80 percent of agricultural GDP and 600 million rural poor people rely on livestock for their livelihoods. Disease outbreaks pose a serious threat to the livestock sector and the impact is often multi-dimensional complicating effective policy response. Among the livestock diseases, parasitic diseases need special mention as it can cause heavy economic losses to the resource poor livestock farmers which are largely ignored because of the sub acute and chronic manifestation of the diseases. In this scenario, the topic of the short course is a priority thrust area and is expected to provide important platform for the exchange of scientific information in the field of diagnosis of parasitic diseases of livestock.

I congratulate the organizers for taking initiative for conducting the short course and it will definitely benefit the scientists/stakeholders from different institutions for better animal health management practices through early diagnosis of parasitic diseases.

I wish this endeavour a grand success.

Place: New Delhi
Date: 26-12-2018

(Joykrushna Jena)

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Dr Ashok Kumar
Assistant Director General (Animal Health)

No.PA/ADG (AH)/2018 **December 19, 2018**

MESSAGE

It gives me immense pleasure that National Institute of Veterinary Epidemiology and Disease Informatics (ICAR-NIVEDI), Bengaluru is organising ICAR-sponsored short course on 'An update on molecular and advanced approaches for diagnosis of parasitic diseases in animals' from 2nd to 11th January, 2019.

Being a tropical country, India is embraced with different parasitic diseases causing severe production losses in animals. Efficient and effective management of parasitic diseases demands accurate early diagnosis of the disease. I am sure that the short course pertaining to diagnosis of parasitic diseases of livestock will help the scientists from different institutes to design the strategies of diagnosis of major parasitic diseases at an early stage of infection thereby the goal of effective management and control of the parasitic disease can be achieved.

I wish the short course all the success and compliment the organizers for selecting the appropriate theme for deliberations.

(Ashok Kumar)

भाकृअनुप–राष्ट्रीय पशुरोग जानपदिक एवं सूचना विज्ञान संस्थान
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Dr. B. R. Shome,
Director (Acting)

MESSAGE

ICAR-NIVEDI, a pioneer research institute under Indian Council of Agricultural Research (ICAR), has been entrusted to conduct Research & Development in the field of Veterinary Epidemiology and animal disease surveillance for the entire country. Precision diagnosis followed by correct intervention is extremely important in controlling the disease and to prevent economic losses caused due to it.

It is a matter of pride for the Institute to host ICAR- sponsored short course on 'An update on molecular and advanced approaches for diagnosis of parasitic diseases in animals' from 2nd to 11th January, 2019 and hope that the deliberations in the program will be beneficial to the participants. This training will create a platform for ICAR-NIVEDI to share our expertise in the field of diagnosis of major parasitic diseases of livestock. I hope this short course will also help all the participants from different institutes to strengthen their capabilities in the area of parasitic disease diagnosis.

I wish the participants a pleasant stay and I wish the short course a great success.

भाकृअनुप–राष्ट्रीय पशुरोग जानपदिक एवं सूचना विज्ञान संस्थान

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Dr. P. P. Sengupta,
Course Director.

Parasitic diseases in animals are one of the major threats to the livestock industry in India. The effective treatment and control of parasitic diseases requires rapid, reliable and highly sensitive diagnostic tests which in turn will help to reduce the economic losses. The most reliable way to diagnose a parasitic infection is by detection and identification of the organism in animals either by blood smear or faecal sample or skin scrapings examination. However, failure to demonstrate or recover a parasite does not exclude the possibility of infection. In this scenario, advanced methods including molecular and serological techniques are highly promising. Molecular techniques especially PCR are highly sensitive in detecting the infection at an early stage and detection of carrier animals. In addition, nucleic acid based detection method will help to differentiate between closely related species. Antibody detection methods are helpful in understanding the prevalence of infection in the country which ultimately helps to strategize the control plan.

This short course is aimed at refreshing and strengthening skills of researchers in the field of diagnosis of major parasitic diseases of livestock. I extend warm welcome to all the participants to our institute and wish a happy, pleasant, comfortable stay as well as fruitful training and interaction.

With warm regards,

(P. P. Sengupta)

LECTURE NOTES

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Introduction to parasitological diagnostic approach for protozoan diseases

P.P.Sengupta & S.S. Jacob

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Diagnosis is very important to treat or control any disease. As the treatment and control vary from one disease to other, hence precision diagnosis is very important; otherwise entire control approach will be jeopardized. The important approaches are:

Clinical History: Getting history by interaction with animal owners may reveal many useful facts which lead to correct diagnosis e.g. intermittent fever in trypanosomosis, grazing aquatic grass land for fluke, insecticide spray in tick infestation etc.

Clinical Symptoms: Clinical signs many times lead to correct diagnosis. But it is useful only in clinical form of disease but not in carrier status.

Post-mortem Examination: Very often in field p.m. examination is given high importance and accepted as authentic. Such lesions are not visible from outside when the animal is alive. Some typical lesions give an indication of diagnosis. Punched out ulcer in abomasums in theileriosis, intestinal/ caecal haemorrhages in different location in poultry coccidiosis, liver in rabbit coccidiosis – are among them.

Blood Smear Examination for blood protozoan parasites:

i) ***Wet blood films***: A small drop of blood is taken on a slide, mixed with anticoagulant and examined under microscopically. In positive case, mobile parasites will be seen.

ii) ***Stained thick smears*****:** On a glass slide a large drop of blood is takenand it is spread with a toothpick or the corner of another slide so that an area of approximately 1.0–1.25 cm in diameter is covered. After air drying, the smear is then dehaemoglobinised, dried, fixed and stained. It is useful in case of tryps when infection is very less.

iv) ***Stained thin smears:***One small drop of blood from suspected animal is placed on a glass slide, with the help of another slide a tongue shaped thin smear is prepared. After drying, the smear is stained with Giemsa's stain for examination under microscope for the presence of parasite.

v) *Lymph node biopsies:* Samples are usually obtained from the prescapular or precrural (subiliac) lymph nodes. Aseptically the lymph node material is taken using needle and syringe. The material is stained and examined microscopically.

b) Concentration methods: In most hosts *T. evansi* can induce mild clinical or subclinical carrier state infections with low parasitaemia in which it is difficult to demonstrate the parasites. In these circumstances, concentration methods become necessary.

i) *Haematocrit centrifugation*: Blood is collected (70 µl) into at least two heparinised capillary tubes. Dry end is sealed by heat or plasticin and is centrifuged sealed end down, at 3000 ***g*** for 10 minutes. The capillary tube is examined and PCV is recorded. This gives an indication on the anaemia of the animal. The capillary tube is then placed in a groove made with pieces of slide glued to a slide. Trypanosomes are large cells that concentrate at the junction between the buffy coat and the plasma, which is observed under the microscope (100–200×).

iii) Buffy coat method: In this method, the blood from suspected animal is taken in capillary tube, then id is centrifuged @3000 rpm for 10 minutes. After that the buffy coat material is taken of the slide and examined under microscope for parasite. This method is useful when parasitaemia is scanty.

iv) Mini-anion exchange centrifugation technique: When a blood sample from animals infected with salivarian trypanosomes is passed through an appropriate anion-exchange column, the host blood cells, being more negatively charged than trypanosomes, are adsorbed onto the anion-exchanger, while the trypanosomes are eluted, retaining viability and infectivity. The sensitivity of this technique can be increased by approximately tenfold by the use of buffy coat preparations rather than whole blood.

Molecular Technique:

Recombinant DNA probes: Specific DNA probes have been used to detect parasites in infected blood or tissue but are not routinely applied. Although molecular methods have a potentially high analytical sensitivity there have been few convincing studies to critically evaluate the diagnostic sensitivity of these tests as compared with other techniques, such as serology.

e) Detection of parasite DNA: Detection of minute amounts of parasite DNA using a PCR assay is a possible means of identifying animals with active infections, and could have the sensitivity and specificity required. A species-specific primers targeting conserved region of the parasite is used. A multiplex PCR sometimes may be very useful for differential diagnosis for many

parasite at one time or different species of same genus. Real time PCR or quantitive PCR can determine the number of DNA copies of the parasite.

Serological tests

a) **Enzyme linked immunosorbent assay (ELISA):** Enzyme linked immune sorbent assay or ELISA has become very popular in diagnosis of parasitic diseases during recent past years. The first step in an ELISA experiment is the immobilization of the antigen/ antibody in polystyrene plate surface of a microtiter plate. After immobilization, a detection antibody is added, which binds to the adsorbed antigen thereby leading to the formation of an antigen-antibody complex. The detection antibody is either directly conjugated to an enzyme, such as horseradish peroxidase (HRP), or provides a binding site for a labeled secondary antibody. In general, ELISAs can be grouped into the four main categories: Direct, Indirect, Sandwich, and Competitive ELISAs.

b). **Fluorescent Antibody Test:** In this test antigen is coated on slide and on this suspected antibody is added and further the reaction is traced with a secondary antibody conjugated with fluorescent agent. Here antibody is detected. This test needs expert observation to distinguish between specific and nonspecific signal. This test is not suitable for screening of large number of samples. Even it being an old test still recommended by OIE standard test for many parasitic diseases e.g. equine piroplasmosis, bovine theileriosis, anaplasmosis etc.

c) Agglutination and Precipitation Tests: When antibodies are mixed with their corresponding antigens on the surface of large, easily sedimented particles such as animal cells, erythrocytes, or bacteria/ parasite, the antibodies cross-link the particles, forming visible clumps. This reaction is termed as agglutination. Agglutination is a serological reaction and is very similar to the precipitation reaction. Both reactions are highly specific because they depend on the specific antibody and antigen pair. The main difference between these two reactions is the size of antigens. For precipitation, antigens are soluble molecules, and for agglutination, antigens are large, easily sedimented particles. Agglutination is more sensitive than precipitation reaction because it takes a lot of more soluble antigens and antibody molecules to form a visible precipitation. To make the detection of soluble antigen and antibody reaction more sensitive, a precipitation reaction can be transformed into an agglutination reaction by attaching soluble antigens to large, inert carriers, such as erythrocytes or latex beads. Two types of agglutination

tests are generally employed - i. Rapid slide/card agglutination test and ii. Plate agglutination test.

The circumoval precipitin (COP) test was used to detect serum antibodies to *Schistosoma mansoni, S. haematobium*, or both species by using eggs of either species of schistosome.

d) Lysis/ neutralization test: In such test on the organism specific antibody is allowed to react and in positive cases lysis of the organism occurs. The immune trypanolysis test, a serological test detecting antibodies and revealing contact with *Trypanosoma brucei* (T.b.*) gambiense*, is increasingly implemented as a remote reference test to refine serological procedures of HAT surveillance under WHO programme and for managing suspect cases that are serologically positive for specific antibodies but in whom trypanosomes are not detected by microscopic observation. TL is restricted to the reference laboratories that can maintain cloned trypanosome populations expressing a particular variable antigen type. The TL test is usually performed on plasma or serum that needs to be kept frozen until use, which impedes storage in the field and transport to the reference laboratory.

e) Dye test: A Sabin–Feldman dye test is a serologic test to diagnose for toxoplasmosis. The test is based on the presence of certain antibodies that prevent methylene blue dye from entering the cytoplasm of *Toxoplasma* organisms. Patient serum is treated with *Toxoplasma* tachyzoites and complements as activator, and then incubated. After incubation, methylene blue is added. If anti-Toxo antibodies are present in the serum, because these antibodies are activated by complements and lyse the parasite membrane, *Toxoplasma* tachyzoites are not stained (positive result); if there are no antibodies, tachyzoites with intact membrane are stained and appear blue under microscope (negative result). The dilution of the test serum at which 50% of the tachyzoites are thin, distorted and colorless is reported as antibody titer of the test serum. The test is highly sensitive and specific with no false positives reported so far.

Drawbacks of this test: 1. Difficulty in maintaining the live tachyzoites. 2. It detects immunoglobulin G(IgG) antibodies, hence cannot differentiate between recent or past infection.

Card agglutination tests

It is well known that certain predominant variable antigen types (VATs) are expressed in common in different strains of salivarian trypanosomes from different areas. This finding was used as a basis for a test for the diagnosis of *T. evansi*, the card agglutination test – CATT/*T.evansi* – was developed at the Laboratory of Serology, Institute of Tropical Medicine,

Antwerp. The test makes use of fixed and stained trypanosomes of a defined VAT known as RoTat 1.2. Both variable and invariable surface antigens take part in the agglutination reaction. In this test whole trypanosome is used as a particulate antigen. This test is useful for easy field screening. But it can cross react with other trypanozoon species (Bajyana Songa et al., 1988).

Latex agglutination tests

A kit is available from ITM, Antwerp. It comprises a lyophilised latex suspension coated with *T. evansi* RoTat 1.2 variable antigens, PBS, positive and negative controls, test cards, plastic spatulas and a rotator. Reconstitute the antigen-coated latex particles using distilled, deionised water.

***In vitro* cultivation:** Some parasites in particular stages are possible to maintain and propagate in vitro and for this suitable media are available, e.g. schizont stages of *T. annulata* in RPMI 1640 / MEM media. Sometime some cell lines are suitable to maintain them say for example macrophage cell lines are helpful to maintain tachyzoites if *T. gondii*. Novy-MacNeal-Nicolle medium (NNN), a bi-phasic media is especially suitable to grow *Leishmania* sp. and *Trypanosoma cruzi*.

Animal inoculationTest: Till date suitable media are not available to cultivate several parasites *in vitro* or it is very hazardous to cultivate them *in vitro*. Sometime small lab animal or large animal are used to propagate them *in vivo*. For *T. evansi* small laboratory rodents are very useful. For dourine rabbits are used for confirmatory purpose. Many times parasites are grown in immune comptomised animals. In equine piroplasmosis, splectomised equines are used to propagate the infection.

Faecal Examination: For diagnosis of gastro-intestinal protozoa, faecal sample examination is useful. This is used in *Eimeria*, *Cryptosporidium* sp. For this purpose quantification of the infection can be done by McMaster counter / Stoll's technique.

Faecal Culture: Sometime treatment of faecal sampleis helpful for diagnosis. In coccidiosis, after sporulation, the examination of sporulated oocysts can yield better diagnosis.

Tissue Digestion: The cyst forming parasites very often remain in tissues like muscles. In such cases the affected tissue needs to undergone digestion by enzymes followed by examination. Tissue digestion helps to reveal parasites. In case of *Sarcocystis* sp. Infection it is used.

Xenodiagnosis: In this procedure vector is allowed to feed on suspected patient, there after the vector is examined for the presence of parasites. This is followed in *T. cruzi* infection. But it is very old, cumbersome and long method.

Imaging: This process is helpful where cyst forming parasite is involved. Generally scanning or radiography is followed. But this should be always confirmed with collateral test. In cerebral toxoplasmosis, such technique may be helpful.

In conclusion, there are several techniques available for diagnosis of protozoan parasites in animals. But the expert is to take decision that which one to adopt in particular case. Moreover, it is always wise to adopt multiple techniques to shape a concrete diagnosis.

Further reading:

1. Mehlhorn,H and Bunnag,D.1988. Parasitology in focus facts and trends. Springer.pp.1-924.
2. Tizard,I. 2012. An introduction to veterinary immunology. Saunders. Pp.1-568.

Introduction to parasitological diagnostic approach for helminth diseases

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Introduction: Parasitic nematodes (helminths) pose a significant and widespread problem for grazing livestock with implications for animal welfare and food production. The diagnosis of helminths largely depends on laboratory based diagnostic techniques especially fecal sample examination in the case of gastrointestinal (GI) parasites as the clinical manifestations are often inconspicuous and non specific. Recent developments in the diagnostic methods and technologies have opened new avenues for improvement of diagnosis of parasitic diseases. There have been several advancements in the microscopy based methods, molecular techniques and serological techniques especially during the last two decades including microarrays, variants of PCR, nanotechnology etc. Increased in the awareness about the livestock parasitic diseases along with technological advances pave way for the development of rapid and highly sensitive diagnostic tests in the field of veterinary Parasitology.

Conventional diagnosis of Helminth infection

- **Faecal sample examination-** Qualitative and Quantitative methods
- **Faecal culture for identification of larvae**: GI parasites
- **Filarial nematodes-** Blood examination for *Setaria*, Skin scraping examination for *Stephanofilaria*
- **Examination of intermediate hosts-** Snails for *Fasciola*, Amphistomes
- **Postmortem examination of representative animals** and parasite identification

Conventional diagnosis of nematode infection is **laborious and expensive, and often not informative** in providing a decision on whether to treat an animal/herd or not.

Fecal egg counts (FEC): The counting of nematode eggs from feces is the commonest method for the diagnosis of gastrointestinal nematode infections. This method is inexpensive, easy to perform and does not require specialized instrumentation, making it suitable for use in most diagnostic settings. Important applications of this technique include estimating infection intensity, estimating levels of contamination with helminth eggs, evaluating the effectiveness of anthelmintics, determining the breeding value of an animal when selecting for worm resistance,

and guiding decisions regarding treatment and control. This method involves mixing feces with a saturated salt or sugar solution (e.g., sodium nitrate or sucrose; specific gravity: 1.1–1.3) to float parasite eggs (with the exception of trematode eggs) on the surface of the suspension. An aliquot of this suspension is aspirated and eggs counted, and the number transformed into eggs per gram (EPG).Various methods have been developed, including the direct centrifugal flotation method (Lane, 1922), the Stoll dilution technique (Stoll,1923), the McMaster method (Gordon and Whitlock, 1939) and the Wisconsin flotation method (Cox and Todd, 1962), of which the McMaster method appears to be most widely used (Nicholls and Obendorf, 1994).

FECs alone should not be used to guide treatment decisions, but should be interpreted in conjunction with information about the nutritional status, age and management of sheep in a flock. However, according to common practice, a FEC of ≥200 EPG is regarded to indicate a "significant" intensity of infection (www.wormboss.com.au). The value of FEC results also depends on the parasite and host species involved. For example, FEC results for adult cattle are of limited diagnostic value, as they do not usually relate to worm burden; FECs in cattle are usually low and require more sensitive flotation methods than for small ruminants; for species of *Nematodirus*, FECs are also regarded to be of limited value, as most pathological damage during infection is caused by the immature stages prior to egg-laying.

Larval culture (LC): Larval culture involves incubating fecal samples containing eggs of strongylid nematodes to allow L1s to hatch and then develop to L3s; the latter are examined microscopically and differentiated morphologically/morphometrically. A number of protocols have been published which differ in the temperatures, times and media used for culture, and the approach of larval recovery. The most widely employed protocol includes an incubation at27 °C for 7 days (MAFF, 1986).

Immunological detection: A number of immunological methods, including those that are based on the detection of an immune response in an infected animal, and those for the detection of parasite antigens, have been developed for the specific diagnosis of parasitic infections. Based on the target molecule (antigen or antibody), such methods can be classified as either "direct" or "indirect".

Direct immunological methods provide direct evidence of an infection and can be based on the detection of parasite antigens present in the circulation of and/or excreta from infected hosts. Parasitic extracts have a complex composition and contain molecules that are sometimes

shared by other parasites (i.e., are cross-reactive). Shared antigenic composition of closely related parasite species represents a challenge, particularly for nematodes, and often leads to cross-reactivity in immunological tests. Also the presence of host materials associated with the parasite can complicate antigen purification and can sometimes interfere with the specificity of a diagnostic assay. Furthermore, the stage of a parasite, used as an antigen source, can influence immunodiagnostic results, as parasites undergo significant structural and biochemical changes during their development. As an example, the antigenic composition of larval stages differs from that of adults and can give rise to variation in diagnostic specificity and sensitivity. Johnson et al. (1996) described an immunodiagnostic assay for the quantitative detection of excretory/secretory parasite antigens in host feces (coproantigens).These authors evaluated the usefulness of this approach in a murine model system using *Heligmosomoides polygyrus*, a trichostrongyloid gastrointestinal nematode related to the common species infecting ruminants. The authors also suggested that the enzyme-linked immunosorbent assay (ELISA) was useful for the detection of parasite antigens in host feces and might have potential for the detection of pre-patent infections. The diagnostic performance of this assay was promising under experimental conditions, but cross-reactivity, fecal components interfering with the reactivity and the loss of antigens in feces were reported (Johnson et al., 1996).

Indirect immunological methods are usually based on the detection of anti-parasite antibodies or cell-mediated immune responses in infected hosts. A variety of methods has been developed and applied to the diagnosis of nematode infections, such as the complement fixation test, indirect immunofluorescence, indirect haemagglutination and ELISA, of which the latter has been most commonly used. However, parasitic helminths possess a huge variety of antigens, and there is limited information on which stages and antigens are actually responsible for eliciting immune responses. Antibody detection from serum has several disadvantages, including that it cannot distinguish between current and past infection, which is a major challenge when evaluating the effects of chemotherapy, does often not reflect infection intensity and sometimes achieves poor specificity, particularly in disease-endemic areas. The detection of anti-*Ostertagia* antibodies in the serum of cattle has been found to be useful for epidemiological and cross-sectional studies, but is only of limited utility for diagnosis on an individual animal basis (Berghen et al., 1993). Although anti-*Ostertagia a*ntibodies are detectable in milk samples by ELISA, there are also some limitations to this approach. The response to parasitic infections is

variable among host individuals, and it has been shown that serum antibody levels can be influenced by factors, such as milk yield, season, mastitis, the number of pregnancies of a cow, stage of lactation and genetic constitution. Also the use of bulk milk samples has been investigated, which has the advantage of being an inexpensive and user-friendly approach. However, bulk milk samples taken only a few weeks apart can show significant variation in test results, depending on calving patterns, number of cows contributing to the milk in a tank (i.e., dilution effect) and their relative milk yields.

Nucleic acid-based methods for diagnosis

Clearly, conventional methods of diagnosis have some limitations, in terms of sensitivity and/or specificity. In addition, they can be time consuming and costly to perform. DNA technologies have enabled the development of new, sensitive and specific diagnostic methods that have found applications in parasitology. The ability to specifically identify and study parasites (irrespective of life-cycle stage) using DNA methods has provided new insights into parasite biology, epidemiology and ecology, and has important implications for the specific diagnosis, treatment and control of parasitic diseases (Gasser, 2006). In particular, methods that rely on the enzymatic amplification of nucleic acids can overcome some of the limitations of traditional approaches (Gasser, 2006). Methods that employ the polymerase chain reaction (PCR) can selectively amplify in vitro target DNA sequences from complex genomes or matrices, and have led to advances in many areas of the biological sciences. PCR involves the heat denaturation of double-stranded DNA, followed by a decrease in temperature to allow oligonucleotide primers to bind (=anneal) to their complementary sequence on sense and antisense strands of the target template. Then, the temperature is increased again to enhance the enzymatic activity of a thermostable DNA polymerase, which extends the complementary strands from the primer sites. These synthesis steps are usually repeated 20–40 times in an automated thermal cycler, resulting in an exponential increase in target DNA copies. The major advantage of this methodology is that it enables the study of parasite DNA from minute amounts of template, which would otherwise be insufficient for conventional analysis. The value of this technology in the field of diagnostic veterinary parasitology lies in its ability to specifically identify parasites, detect infection and analyze genetic variation, which are particularly important, given the increasing problems of AR in parasitic nematodes.

Genetic markers for specific identification or detection

The key to developing a reliable PCR method for the specific diagnosis of infection is the definition of one or more suitable DNA targets (genetic marker or locus) based on DNA sequencing. Since different genes evolve at different rates, the DNA region selected should be sufficiently variable in sequence to allow the identification of parasites to the taxonomic level required. For specific identification, the target DNA should display no or minor sequence variation within a species and differ sufficiently in sequence to consistently allow the delineation among species. In contrast, for the purpose of identifying population variants (subspecies, genotypes or "strains"), a considerable degree of variation in the sequence should exist within a species. A range of target regions in the nuclear and mitochondrial genomes have been employed to achieve the identification of parasites to species or sub-specific genotypes (Gasser, 2006). In nuclear ribosomal genes and spacers, there is often less sequence variation among individuals within a population and between populations, which makes them suitable as specific markers. Hence, in the case of genetic markers for the specific identification of strongylid nematodes of livestock, most of the focus has been on employing nuclear ribosomal DNA (rDNA). Although some success was achieved with other DNA targets, most studies have consistently shown that the first (ITS-1) and second (ITS-2) internal transcribed spacers (ITS) of nuclear rDNA provide reliable genetic markers for the specific identification of a range of strongylid nematodes of livestock, including species of *Haemonchus*, *Teladorsagia* and *Ostertagia* (abomasum); *Trichostrongylus* (abomasum or small intestine), *Cooperia*, *Nematodirus*, *Bunostomum* (small intestine); *Oesophagostomum* and *Chabertia* (large intestine); *Dictyocaulus*, *Protostrongylus* and *Metastrongylus* (lung). A comparison of the ITS sequences from a range of strongylid nematodes has shown that the ITS-1 (364–522 bp) is usually larger in size than the ITS-2 (215–484 bp) (Chilton, 2004). For instance, the ITS-1 region of *Ostertagia ostertagi* and *O. lyrata* (801 bp) is longer than that of other trichostrongylids, including congeners, due to the presence of an internal 204 bp region, which is repeated twice. No major differences have been detected among species of *Teladorsagia*/*Ostertagia* in the lengths of their ITS-2 sequence (Chilton et al., 2001; Stevenson et al., 1996). The G+ C content (39–50%) of the ITS-1 sequence of species studied is usually greater than of their ITS-2 (29–45%). The ITS-2 sequences of some species can be relatively A+T-rich (60–70%), which may relate to structural aspects of the precursor rRNA molecule. In addition, studies to date, show that the magnitude of sequence variation in both the ITS-1 and ITS-2 within a species is less (usually b1.5%) than the levels of sequence differences

among species (Gasser, 2006), providing the basis for the specific identification of strongylids and diagnosis of infections.

RT-PCR assays for the diagnosis of strongylid nematode infections

In spite of promising results of RT-PCR for the diagnosis and quantification of selected protozoan and metazoan parasites, to date, relatively little research has focused on its use for the diagnosis of strongylid infections of livestock (Gasser, 2006). There have been attempts to use RT-PCR for the specific diagnosis and/or quantification of helminth eggs or larvae from the feces from infected hosts. First efforts were made by von Samson-Himmelstjerna et al. (2002, 2003), who developed RT-PCR assays for the diagnosis and quantification of ovine gastrointestinal nematodes, including *H. contortus*, *O. leptospicularis*, *T. colubriformis* and for small strongyles (cyathostomins) of horses. However, these assays were used for the identification of larval or adult nematodes only, which limited their utility for routine diagnostic applications

Prospects for field-based assays

PCR-based technologies have become central to the diagnosis of parasitic and other infectious diseases. However, most PCR methods are laboratory based and often require relatively expensive and specialized instrumentation and reagents, a clean laboratory to avoid contamination during processing and testing and skilled operators with some technical knowledge in molecular technologies. Therefore, most PCR assays are not applicable to use in the field, which represents a limitation for application in resource-poor countries or in situations where a rapid ‘on-the-spot’ diagnosis is required. Recent developments provide possibilities for the miniaturization and automation of devices for diagnostic testing. The time required to perform PCR (typically 90–120 min) depends, to a large extent, on the ability of the instrument to cycle rapidly through the denaturation, annealing and extension steps. By reducing the thermal mass of the instrument, the total reaction times can be significantly shortened. Two different parameters can be altered to achieve this: (i) the physical dimensions of a system can be reduced to reduce the thermal mass of the instrument, and (ii) a sample can be moved through multiple reaction zones which are held at specific temperatures required for the in vitro amplification of nucleic acids. The latter approach allows the heating or cooling of small fluid elements which move through different temperature zones within 100 ms, thus allowing ultra-rapid reaction times and making the functional integration of PCR into micro-chips possible. Obeid et al.

(2003) developed such a microchip that demonstrated efficient amplification of DNA within 5 min and, additionally, provides a separate channel for reverse transcription, in which RNA samples can be transcribed into DNA before entering the PCR zone of the chip for amplification. Furthermore, the continuous injection of small sample volumes (1–2 μl), separated by water and air plugs, allows the simultaneous amplification of multiple samples without cross-contamination. Isothermal amplification methods provide prospects for the design of simple, portable and low-energy consuming operating systems. Some of these methods include nucleic acid sequence-based amplification (NASBA), loop-mediated isothermal amplification (LAMP), helicase-dependent amplification (HDA), rolling circle amplification (RCA) and strand displacement amplification (SDA). Advantages of such methods seem to be that they reduce costs, simplify the use of amplification reactions by eliminating laboratory equipment, prevent contamination and provide the potential to run several reactions in parallel. Furthermore, miniaturized test systems can be combined with integrated steps of sample preparation and detection of amplification products, thus providing scope for the design of monolithic diagnostic systems, also referred to as micro-total analysis systems (μTAS) or labs-on-a-chip (LOC). Although, these technologies appear to be in their early phase of development and require further research to translate into practical diagnostics, there are some reports demonstrating their utility. For example, Liu et al., 2011 developed a point-of-care diagnostic system, which is equipped with an integrated membrane for isolation, concentration and purification of nucleic acids. In this system, the amplification process is carried out using a LAMP procedure coupled to real-time detection of amplicons using a fluorescence reader. The authors assessed their system for the detection of human immunodeficiency virus (HIV-1) from oral fluids and demonstrated a detection limit of less than 10 HIV particles (Liu et al., 2011). A number of LAMP assays have already been reported for a range of metazoan parasites, including taeniid cestodes, *Fasciola* spp., *Opisthorchis viverrini*, *Paragonimus westermani* and *Angiostrongylus cantonensis* and *Wuchereria bancrofti*. However, these assays are still laboratory-based and, to date, there is no point-of-care diagnostic system for routine use. These recent achievements indicate some potential for novel diagnostic systems with prospects for rapid, field-based diagnosis.

Future diagnostic applications of advanced sequencing technologies

Further advances in molecular diagnostics are expected from the rapidly developing sequencing technologies. In the past, studies that investigated the diversity of microorganisms in a natural

sample involved the cloning and subsequent Sanger sequencing of selected genes (commonly 16S rDNA) to produce profiles of diversity in environmental samples. These so-called metagenomic studies (i.e., the study of genetic material recovered directly from environmental samples) showed that cultivation-based methods are unable to detect the vast majority of microorganisms and have allowed novel insights into a previously hidden diversity of microbial life. Following the rapid reduction in cost of DNA sequencing and the development of high-throughput sequencing technologies (e.g., 454 pyrosequencing, Illumina- and SOLID sequencing platforms), these technologies have become accessible to many research groups and have enabled the direct sequencing of microbe and parasite genomes from environmental samples. However, these novel approaches of DNA sequencing create read lengths which are significantly shorter than those produced by Sanger sequencing and a much larger number of sequence reads, ultimately leading to the generation of large amounts of data for analyses. Analyses of such data sets to obtain biologically meaningful information and the need for enhanced computing power can represent a bottleneck to this approach. Nonetheless, a number of studies show the value of metagenomic approaches for assessing the diversity of microbial communities in the gut of humans and other mammals or in the marine environment and have led to the discovery of large numbers of previously unknown microorganisms. Another technological advance is DNA sequencing using nanopores. This type of analysis is emerging and involves the use of voltage to drive molecules through a nano-scale pore in a membrane between two electrolytes. This allows the analysis of charged polymers (single-stranded DNA, double-stranded DNA or RNA) by monitoring the change of the ionic current as single molecules pass through it. Nano-pore sequencing has the advantages that it does not require the labeling of nucleotides or amplification prior to sequencing, can be applied to single molecules and is capable of high throughput DNA analysis. Further benefits are that it is low cost, requires small reagent volumes and generates long reads, which appears to make it suitable for de novo sequencing. It has been proposed that nano-pore-based diagnostic tools could detect target molecules at extremely low concentrations and from minute sample volumes, detect simultaneously multiple biomarkers or genes, eliminate the need for time-consuming amplification and conversion steps, thus providing a rapid analysis at low cost. In addition to the sequencing through nano-pores, with the aim of rapid and affordable DNA sequencing, a number of other approaches have been developed, including the single molecule evanescent field detection of sequencing-by-synthesis in arrays of nano-

chambers (Pacific Biosciences), sequencing by ligation on self-assembled DNA nano-arrays (Complete Genomics), and the detection of H+ions released during sequencing-by-synthesis on silicon field-effect transistors from multiple polymerase template reactions. Clearly, the rapid emergence of a range of exciting sequencing technologies provides new prospects for diagnostic applications. Current evidence suggests that some of these advanced technologies will change the face of molecular diagnostics in the near future.

Conclusions

The accurate diagnosis of gastrointestinal nematode infections of livestock underpins investigations of the biology, ecology and epidemiology of parasites and supports the monitoring of emerging problems with anthelmintic resistance (AR). Current, routinely used methods of diagnosis rely on the detection or morphological identification of the infective stages (eggs and/or larvae) of these nematodes in host feces. Until recently, these traditional techniques, which can be time-consuming and laborious, have not undergone any substantial technological advancement. As eggs and larvae of numerous genera and species of nematodes infecting livestock lack distinctive morphological features, traditional approaches are not usually able to achieve a species- or even genus-specific diagnosis in the live animal, making it challenging to conduct reliable studies of the biology, epidemiology and ecology of parasites, unless expensive and laborious post mortem investigations are carried out. This situation has also hampered investigations of the occurrence and distribution of AR in strongylid nematodes of livestock, which represents a global problem. Advances in PCR-based methods and the availability of specific genetic markers in the internal transcribed spacers of nuclear rDNA have provided the opportunity of developing enhanced PCR-based tools for diagnosis.

References:

1.Berghen P, Hilderson H, Vercruysse J, Dorny P. Evaluation of pepsinogen, gastrin and antibody response in diagnosing ostertagiasis. Vet Parasitol 1993;46:175–95.

2. Chilton NB. The use of nuclear ribosomal DNA markers for the identification of bursate nematodes (order Strongylida) and for the diagnosis of infections. Anim Health ResRev 2004;5:173–87.

3. Cox DD, Todd AC. Survey of gastrointestinal parasitism in Wisconsin dairy cattle. J Am Vet Med Assoc 1962;141:706–9.

4. Gasser RB. Molecular tools—advances, opportunities and prospects. Vet Parasitol 2006;136: 69–89.

5. Gordon HM, Whitlock HV. A new technique for counting nematode eggs in sheep faeces. J CSIR 1939;12:50–2.

6.Johnson MJ, Behnke JM, Coles GC. Detection of gastrointestinal nematodes by a coproantigen capture ELISA. Res Vet Sci 1996;60:7-12

7. Lane C. The mass diagnosis of ankylostome infestation. (Part I). Trans R Soc Trop Med Hyg 1922;16:274–315.

8.MAFF. Manual of Veterinary Parasitological Laboratory Techniques. London, UK: Her Majesty's Stationary Office; 1986. p. 20–7

9.Nicholls J, Obendorf DL. Application of a composite faecal egg count procedure in diagnostic parasitology. Vet Parasitol 1994;52:337–42

10. Obeid P, Christopoulos T, Crabtree HJ, Backhouse C. Microfabricated device for DNA and RNA amplification by continuous-flow polymerase chain reaction and reverse transcription-polymerase chain reaction with cycle number selection. Anal Chem 2003;75:288–95.

11. Stoll NR. Investigations on the control of hookworm disease. XV. An effective method of counting hookworm eggs in faeces. Am J Epidemiol 1923;3:59–70

12. von Samson-Himmelstjerna G, Harder A, Schnieder T. Quantitative analysis of ITS2sequences in trichostrongyle parasites. Int J Parasitol 2002; 32:1529–35.

13. von Samson-Himmelstjerna G, Buschbaum S, Wirtherle N, Pape M, Schnieder T. TaqMan minor groove binder real-time PCR analysis of beta-tubulin codon 200 polymorphism in small strongyles (Cyathostomin) indicates that the TAC allele is only moderately selected in benzimidazole-resistant populations. Parasitology 2003;127:489–96.

14. Liu C, Geva E, Mauk M, Qiu X, Abrams W, Malamud D, et al. An isothermal amplification reactor with an integrated isolation membrane for point-of-care detection of infectious diseases. Analyst 2011;136:2069–76.

Parasitological approaches for identification of arthropods of veterinary importance

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Introduction: India is one of the top ten mega diversity nations in the world in terms of insect diversity with 7.10% of the world's insect fauna. Insects are the most diverse group on the planet representing more than half of all known living organisms. Importance of insects for man and animals is both direct and indirect. Direct effects on man and animals include annoyance and worry, blood loss and anaemia, predisposition of the bite wounds to infestations for myiasis and mange. It also includes damage of tissue due to insects' invasion, contamination of food by houseflies and cockroaches, inoculation of poisonous substances and cause of allergy, flea allergy dermatitis, venomousbites, entomophobia or delusory parasitosis and undermining host defenses. Indirect effects on man and animals is best expressed by their role as vectors of many diseases.

Arthropods are bilaterally symmetrical dieoecious animals, have a chitinous exoskeleton and a body divided into separate rings or segments, each of which may bear jointed appendages. They possess a complete digestive tract, except in a few species, with mouthparts adapted for biting, chewing or sucking. The arthropods of veterinary importance belong to class: Insecta (fleas, lice, flies, bugs), Arachnida (ticks and mites), Pentastomida (tongue worms) and Crustacea (copepods, amphipods, crabs, crayfishes). Insects and other arthropods are of great parasitological importance with respect to humans and animals. They may be parasitic, living more or less permanently on or in the body of the host, and they may serve as intermediate hosts in the life cycle of parasites and vectors of pathogens.

Collection of arthropods: The proper collection of arthropodsis pre requisite to any subsequent research or control programmes involving these organisms. It is important to know what kinds of arthropods are of potential significance to animal health , their location and population density. Collection and preparation methods for arthropods vary depending upon the species or group of arthropods concerned as well as the objectives of the collection. For collection of arthropods basic but necessary entomological equipment is an insect net, a killing jar and a pinning box or vials for keeping specimens. For collecting adult mosquitoes or other small insects, a suction

tube, or aspirator is useful. Dipterans such as mosquitoes, biting midges, phlebotomines, tabanids and others can be collected by using traps specially designed for them. Some collecting devices employ an attractant or lure to induce arthropods to enter trap.

Arthropods such as wolf spiders, ticks, beetles and cockroaches that run or crawl over the surface of the ground can be collected in any of various types of pitfalls. The tick drag is used to collect unengorged ticks and chiggers from the ground. The collection of ectoparasites from living small mammals or birds can be facilitated by hand holding the host over a white enamel collecting tray and brushing its pelage vigorously with a nitcomb or stiff bristled brush (old tooth brush is quite suitable), a few puffs of pyrethrin dust directed into the fur or feathers will facilitate the removal of most ectoparasites. Often the arthropods collected are needed alive or to be preserved freshly so that they can be tested for presence of pathogenic organisms and for the source of any blood meals they contain.

Preservation of arthropods: is done by two methods ie liquid and dry preservation. Ticks, mites, fleas, cockroaches, spiders, scorpions and soft bodied larvae of insects are usually preserved in 70%ethanol. Formalin at 2 to 10% can be used. When arthropods are placed in alcohol they tend to curl up, making identification difficult. Under laboratory conditions this can be avoided by killing fresh specimens in water at about 80^0C, they are then transferred to preservative. A temporary label should be written with pencil on paper and placed inside the tube. After examination a label should be written in waterproof carbon ink on thin cardboard, to be placed permanently inside the tube. It should have the details of site and date of collection, and collectors name. The adults of larger insects such as tsetse or blow flies can be preserved dry; this has the advantage of preserving setae, spines and colour better for identification. Mosquito adults are not easy to handle for dry preservation but this may be essential to retain the pattern of scales and setae for identification. When the insects are still soft they are fixed on a pin through the thorax slightly to the right of the midline. The legs and wings are arranged in a convenient position. The pin is also passed through a label and is fixed in a cork board in an entomological specimen box or in a jar. Pinned insects can be stored in standard insect boxes (Schmitt boxes).

Clearing and mounting of arthropods: Parasitic mites such as Notoedres, Otodectes, Sarcoptes, etc., are usually deeply embedded in the host tissue and are therefore often lost or

damaged when an attempt is made to separate them. For collecting them, a scalpel or knife blade is sterilised by passing it through a flame and then cooled by dipping it in water. Mites are collected more easily if the edge of the scraper is first dipped in acetic acid glycerine (1% glacial acetic acid in glycerine). A fold of skin showing lesions is held between thumb and forefinger and the crest of the fold scraped until the lymph begins to ooze. Care must be taken to avoid drawing blood. Transfer scrapings from scalpel, using a rotary motion, into a drop of acetic-glycerine on a slide. Add a coverslip and additional acetic-glycerine, if necessary, and observe under the lower power of a compound microscope. The material may be stored in the acetic-glycerine indefinitely, and mites may be separated at leisure. Add a small crystals of thymol to discourage growth of molds. Separate parasites with a very fine dissecting needle or finely drawn pipette equipped with a rubber bulb. This is under a dissecting microscope in good light. After mites have been separated from the debris, place them on a slide in one of the several well established chloral-gum preparations.

Specimens can be directly mounted on microscopic slides when Hoyer's medium is used. Small insects such as biting midges, mosquitos, phlebotomines, males and unengorged ticks, fleas etc can be cleared by carbolic acid (Liquified phenol) after clearing a drop of phenol-balsam mixture (1:1 ratio of liquid phenol and canada balsam) was placed on a microscope slide. Then the specimen from the phenol solution was transferred to the slide and mounted.(Wirth and Martson (1967). Mites and smaller insects such as fleas, lice, ceratopogonids and phlebotomines can be examined directly from preservation in liquid in a tube, but if correctly mounted on a microscope slide important features for identification may become visible. There are three basic methods: temporary mounts in water soluble, permanent mounts in water soluble media, permanent mounts in water insoluble media.

Freshly collected mites in glycerol or oil can be mounted directly on a microscope slide, a coverslip placed over and the specimen examined as a temporary mount. For permanent mounting the specimens are first fixed by immersion in ethanol or formalin for several hours. A convenient water soluble medium is the Berlese’s chloral gum medium . Permanent mounting in water insoluble media gives the best results. Heavily sclerotizedor blood engorged specimens may be cleared by piercing the abdominal region with a small insect needle while the specimen is still in the preservative, and then transferred to water. Replace the water with 10% KOH and

leave for 12 to 24hrs or until the specimen becomes lighter on colour. The time in KOH can be shortened considerably by heating the solution containing the specimen for about 10min. Small insects are cleared in carbolic acid, then should be dehydrated for one hour in each of the following; 70%, 85%, 95% and absolute alcohol. They are then immersed in a clearing agent such as beech wood creosote, clove oil or xylol before being mounted in Canada balsam, phenol balsam or DPX.

Identification of insects: is essential from the point of the vector potentiality, invasive species, accelerated extinction rate, biodiversity and conservation, for quarantine and biosecurity, in forensic entomology etc. Insects can be identified by morphological and molecular methods. Morphological identification of insects is done on the basis of charecterisitc features. E.g: Characters for identification of *Culicoides* spp:- Wing markings, antennal ratio & palpal ratio, distribution of antennal coeloconica in female, form of sensory organs on 3rd palpal segment, form of whole 3rd palpal segment, number & form of spermathecae, form of male genitalia, appearance of leg & thorax.length of body, length and breadth of wing, costal ratio (Wirth and Hubert, 1989). Likewise for individual insects there morphological keys used for identification. Morphological identification of insects can be done on the basis of their morphological characters and keys. According to Mayr and Ashlock (1991) it took 200 years for the taxonomists to describe 1.7 million species which accounts for 10% of the total number of species prevalent. They estimated that 15,000 taxonomists working for centuries can complete the task of identifying the remaining 90% of species. Phenotypic plasticity and genetic variability, morphologically cryptic taxa/biotypes keys are effective for a particular life stage, sympatric and allopatric variations, need for specialist, need for holotype, and time consumption are some of the limitations.

Molecular method of identification aids in faster identification of species with increased sensitivity and specificity. Closely related species in complex groups can be differentiated. Different molecular methods for identification of insects include protein and nucleic acid methods. Protein method relies on allozyme electrophoresis. Nucleic acid methods include DNA hybridization, PCR, RAPD, RFLP, Microarray, Microsatellite and AFLP (Loxdale and Lushai 1998). DNA barcoding has been widely used in species identification and biodiversity research (Kim*et al*., 2012).

Finally, although modern interactive versions represent a major advance, the use of keys often demands such a high level of expertise that wrong identification is common. Morphological method is tedious, time-consuming, requires holotype comparison, large number of insects cannot be identified in a short period of time and it requires taxonomic expertise. The morphological similarity in females of certain species complex of insects is a challenge for identification. The molecular method of identification is being advocated for identification, which is faster and has increased sensitivity and specificity.Therefore this write up highlights on the use of these techniques.

Allozyme electrophoresis: Most carrion-breeding species of blowflies belong to the genus *Calliphora.* The morphological similarity of the immature stages of these species requires correct identification and is a challenge for forensic entomologists. A study investigated the potential of allozyme analysis to assist with this task. Molecular profiles of third-instar larvae and adults representing four of these carrion-breeding species, *Calliphora stygia, C. dubia, C. hillihilli,* and *C. vicina,* were compared at 42 allozyme loci. The two life stages were found to display almost identical allozyme profiles in each species (93% of loci were expressed in both life history stages), enabling the reliable identification of larvae in these four species. This study indicated that allozyme analysis would also be suitable for rapid, species-level identification of the larvae of six other carrion- breeding *Calliphora* species occurring in southern Australia (Wallman and Adams 2001).Four populations of *Culex tritaeniorhynchus* were analyzed for morphological and allozyme variation whichindicated the influence and selection by the environmental conditions (Kanojia et al. 2009).

Hybridization method:A simple method was developed for species identification of mosquitoes in the *Anopheles dirus* complex found in Thailand using horseradish peroxidase-labeled DNA probes and a chemiluminescent detection system. Species-specific DNA probes for *Anopheles dirus*, species B, C, and D, detected 1-5 ng of target DNA, a sensitivity that was comparable with the radioisotopic detection system. Identification of individual mosquitoes was performed by dot-blot analysis of crude mosquito DNA. The method allowed identification using third or fourth instars as well as the adult head, thorax or abdomen. This technique successfully identified the sibling species of the *A. dirus* complex in field collections (Audtho et al. 1995).

Polymerase chain reaction (PCR):*Culicoides* species were identified by genus-specific PCR and *C. imicola* specific PCR by targeting ITS-1 region of rDNA (Sossah et al.2004).Similarly, PCR has been used for identification of various insects, Kiehl et al. (2009) found that *Culicoides obsoletus* and *C. pulicaris* are definitive species and they probably belong to so called complexesby the molecular biological study on their ITS-1, ITS-2 and 18S rDNA characteristics differentiated*C. pulicaris* females morphologically and by size they can be significantly from different from *C. obsoletus.*Balczun et al. (2009) reported that since females of the complex of *Culicoides obsoletus* are difficult to be identified on morphological criteria, a PCR based strategy for targeting the mitochondrial Cytochrome Oxidase subunit I to differentiate between the species *Culicoides obsoletus* and*C. scoticus* was successful.Stephan et al. (2009) did PCR identification of *C. dewulfi*a potential vector of bluetongue in Germany. Sperling et al. (1994) used mitochondrial DNA, mitochondrial cytochrome oxidase I (COI), cytochrome oxidase II (COII) and tRNA leucine genes of blowflies *Phormia regina, Phaenicia sericata* and *Lucilia illustris* which were amplified using PCR and followed by direct sequencing and found that there were nucleotide differences in the DNA sequences between these three species which could be used to differentiate their immature larval stages.Mazzanti et al. (2010) demonstrated that PCR could successfully amplify the mtDNA from empty puparial case and also its fragments. They could also correctly determine the eight Dipteran species (*Calliphora vicina, Sarcophagidae crassipalpis, Phormia regina*, *Phaenicia sericata, Sarcophaga argyrostoma, Calliphora vomitoria, Chrysomya megacephala, Synthesiomyianudiseta)* through the amplified DNA from pupal case. This finding is particularly important as empty pupal cases left after adult emergence or fragments of it are commonly found on the corpse or in the area surrounding the corpse.

Molecular identification of five Indian muscid flies (Musca domestica, Musca sorbens, Musca crassirostris, Stomoxys calcitrans and Haematobia irritans) has been attempted on the basis of mitochondrial COII gene. Sequences of 500-520 bp were analysed and differentiated muscid fly species (Singh and Achint, 2017). Genetic characterization of Rhipicephalus (Boophilus) annulatus by COI and ITS2 sequences could provide suitable genetic markers for discrimination of this species (Ronaghi et al., 2015). Morphologically identified Demodex mite was subjected to PCR amplification and DNA sequencing of for molecular confirmation of Demodex cati in a cat by targeting 16S rRNA DNA (Bernstein et al., 2014).

Multiplex PCR: The mitochondrial cytochrome oxidase subunit I (COI) DNA sequences have been used for taxonomic resolution and species identification of all species within *C. obsoletus* and *C. pulicarus* complexes. Levels of DNA sequence divergence were sufficiently high between species to allow the design of species-specific PCR primers for identification of members of the *C. pulicaris* complex or in a multiplex PCR to identify members of the *C. obsoletus* complex. This approach provided a valuable diagnostic tool for monitoring species composition in mixed field collections of *Culicoides* (Nolan et al. 2007).The molecular identification of five morphologically related subgenus Stergomyia mosquito- *Aedes aegypti*, *Ae.albopictus*, *Ae. riversi*, *Ae. flavopictus*and *Ae. daitensis*was done by targeting ITS region. First genus specific amplification was done by using common primers and the PCR products were sequenced and species specific primers designed. The five species were identified specifically with conditions for multiplex PCR optimized in a single reaction (Higa et al. 2010).

Real-Time PCR: Real time PCR was conducted by using green fluorescence dye (sybr green)for distinguishing spp. of *C. obsoletus* complex, followed by comparison of derivative melting curves in post amplification phase that is by analyzing species specific pattern of dissociation curves, it was possible to identify 3 different spp. of the complex. After PCR a melting curve analysis was conducted with a denaturing phase from 60^0c-95^0c according to the standard ramp rate. The melting curve plot obtained in presence of SYBR green clearly distinguished 3 spp. Tm value for each spp. is 73.1^{o}c for *C. obsoletus*, 79.2^{0}C for *C. scoticus* 72.4^{0}C and 79.2^{0}C for *C. montanus* (Monaco et al. 2010).This real-time PCR assay was first performed on 10-fold serial dilutions of purified plasmid DNA containing specific *C. imicola* ITS-1. The results indicated that this real-time PCR assay holds promise for monitoring *C. imicola* population in both surveillance and research programmes because of its good specificity (92%) and sensitivity (95%) (Cetre-Sossah et al. 2008).

TaqMan SNP genotyping proved to be a sensitive and rapid method for identification of *An. gambiae* and *An. arabiensis*, with a high success rate, specific results, and congruence with the standard PCR method (Walker et al. 2007).TaqMan assay was developed to distinguish between the main malaria vectors (*An. Arabiensis* and *An. gambiaes.s.*) and the non-vector *An. Quadriannulatus* (Bass et al. 2007).

Random amplified polymorphic DNA (RAPD): Based on the need for DNA sequence information, PCR assays are limited in their application. Therefore one of technique which requires no DNA probes and sequence information for the species identification is RAPD.

Entomological surveys in the state of Maranhao have recorded morphologically distinct populations of *Lutzomyia longipalpis.* Some populations have one pair of spots (1S) on the fourth tergite, while others have two pairs (2S) on the third and fourth tergites of males. RAPD-PCR reactions were performed individually for each of the twenty primers tested. Of the twenty primers that could potentially be used to analyze populations of *L. longipalpis,* only four *(OPA-3,* OPA-4, OPA-9 and OPA-15) were selected from preliminary RAPD-PCR tests. They produced informative, reproducible and clear information on the genetic variation for the specimens and populations studied. The results of RAPD analysis showed a clear separation between the populations with one and two pairs of spots (Silva et al. 2011).

Restriction fragment length polymorphism (RFLP): Isolation of sufficient DNA for RFLP analysis is time-consuming and labor intensive. However, PCR can be used to amplify very small amounts of DNA, usually in 2-3 hours, to the levels required for RFLP analysis. Therefore, more samples can be analyzed in a shorter time.*Phlebotomus argentipes* remains the only known vector although a number of sand fly species are prevalent in this area. Genotyping of sand flies distributed in a VL endemic area was developed by a PCR and restriction-fragment-length polymorphism (RFLP) of 18S rRNA gene of sand fly species. Using the RFLP-PCR analysis with *Afa*I and *Hin*fI restriction enzymes, *P. argentipes, P. papatasi*, and *Sergentomyia* species could be identified (Alam et al. 2012).*Anopheles* mosquitoes are routinely identified using morphological characters of the female that often lead to misidentification due to interspecies similarity and intraspecies variability therefore PCR-RFLP-ITS2 assay has been applied for accurate discrimination of anophelines. PCR-RFLP-ITS2 assay performed with *AluI, FspI or DraIII.* The ITS2-PCRRFLP assay proved valuable for discriminating anopheline species (Cienfuegos et al. 2011).Sperling et al., 1994 used RFLP-PCR of mtDNA to separate species of economically important ermine moths and forensically important Diptera. Combinations of the restriction enzymes *Dde*I, *Hin*fI and *Sau*3AI provided diagnostic bands for identification of the ten species from three families of Diptera (Calliphoridae, Muscidae and Sarcophagidae) (Ratcliffe et al. 2003).

Microarray: The internal transcribed spacer 1 (ITS1) gene sequences of 55 *Culicoides* belonging to 13 different species were determined and used, together with 19 *Culicoides* ITS1 sequences sourced from GenBank, to design species specific probes for the microarray test. This test was evaluated using the amplified ITS1 sequences of another 85 *Culicoides* specimens, belonging to 11 species. The microarray test successfully identified all samples (100%) of the Obsoletus group, identifying each specimen to species level within the group. This method has several advantages over existing polymerase chain reaction based molecular tools, including possible capability for parallel analysis of many species, high sensitivity and specificity, and low background signal noise. Hand-spotting of the microarray slide and the use of detection chemistry render this alternative technique affordable and feasible for any diagnostic laboratory with PCR facilities (Deblauw et al.2011).

Microsatellite: Microsatellites have proved to be very useful as genetic markers, as they seem to be ubiquitous and randomly distributed throughout most eukaryote genomes. The isolation and identification of microsatellite sequences from multiple genomic libraries for *A. aegypti*, identified 6 single-copy simple microsatellites from 3 plasmid libraries enriched for (GA)n, (AAT)n, and (TAGA)n motifs from *A. aegypti*. In addition, 5 single-copy microsatellites from an *A. aegypti*cos mid library were identified. Genetic map positions were determined for 8 microsatellite loci. These markers greatly increase the number of microsatellite markers available for *A. aegypti* and provide additional tools for studying genetic variability of mosquito populations. Additionally, most *A. aegypti* microsatellites are closely associated with repetitive elements which accounts for the limited success in developing an extensive panel of microsatellite marker loci (Chambers et al. 2007).

AFLP (Amplified fragment length polymorphism): Advantages include it is as a fingerprinting technique replacing RFLP, it is highly polymorphic, highly reproducible and can identify through the presence of even a fragment. Lall et al. (2010) used AFLP for analysis of closely related wild and captive Tsetse fly.

DNA barcoding: Several researchers have suggested the use of DNA barcoding in taxonomy as a method to achieve rapid species descriptions in the context of the current biodiversity crisis (Hebert et al. 2003; Ball and Armstrong 2006). DNA barcoding is the use of a short standardized DNA sequence in insects, a 658 bp fragment of the mitochondrial cytochrome *c* oxidase (*COX I*)

gene to identify and assign unknown specimens to species besides facilitating the discovery of new species. This tool is widely accepted all over the globe from hard-core taxonomists' to graduate molecular biologists and also well received by governmental and nongovernmental organizations to catalogue all the species on our planet. With the advent of molecular biology and molecular tools, identification of life forms, including insects has become quick, precise, and easy (Jalali et al. 2015). DNA barcoding has been used for confirmation of morphologically identified insects successfully (Hebert et al. 2003; Kim et al. 2012; Rivera et al. 2009). The mitochondrial gene Cytochrome *c* oxidase I (COI) could serve as the core of a global bio identification system for animals. COI identification system can provide a reliable, cost-effective and accessible solution to the current problem of species identification (Hebert et al. 2003).

The approximate number of described insect species in India is 59,000; however, the number of barcodes generated from India is 4.6 % of known species, while the corresponding global scenario is about 16 % of described species. A lot of work remains to catch up with the world scenario. In order to speed up taxonomic identification, DNA barcoding is now been considered as an alternative tool for insect biodiversity identification in India and the world (Jalali et al. 2015). Mitochondrial genome of animals is a better target for analysis than the nuclear genome because of its lack of introns, its limited exposure to recombination and its haploid mode of inheritance (Saccone et al. 1999). Mitochondrial DNA is used for DNA barcoding because Mt DNA is much smaller than nuclear DNA, sequencing is easy. Mt. DNA has fast mutation rate with a significant variation between species and less variation among species. It is less prone to insertions, deletions and other large scale of rearrangements which spoils the generation of barcode. It is very stable with little or no degradation in museum specimens and easy to design primers, easy alignment having short segments with less cost to sequence (Mandal et al. 2014).

The mitochondrial cytochrome oxidase I (COI) gene has been proposed as standard DNA barcoding marker for the identification of organisms. COI provides an ideal species identification marker in insects, due to lack of introns, simple alignment, limited exposure to recombination and the availability of robust primer sites. Sequence variation in this region generally shows large interspecific, but small intraspecific divergence. COI is used for DNA barcoding as it is the most popular barcode used worldwide and very efficient for species

identification. It is highly conserved DNA sequence within species, its alignment process is the not difficult- protein coding region and it is easy to isolate from wide range of organisms.

DNA barcoding uses short DNA sequence instead of whole genome and it is used for eukaryotes. The short DNA sequence (658bp) is taken from standard region of genome to generate DNA barcode. DNA barcode is short DNA sequence made of four nucleotide bases A (Adenine), T (Thymine), C (Cytosine) and G (Guanine). Each base is represented by a unique color in DNA barcode (A=Green, T=Red, C=Blue and G=Black). The short DNA sequence is generated from standard region of genome known as marker. This marker is different for various species like COI cytochrome c oxidase 1 for animals, matK for plants and Internal Transcribed Spacer (ITS) for fungus. COI (Cytochrome C Oxidase 1) present in mitochondrial gene in animals proposed by Paul Hebert and recognized by International Barcode of Life (IBOL) as official marker for animals, because of its small intra specific and large inter specific differences in animals.

The process of DNA barcoding involves two basic steps: first step is to build a barcode library of identified species and second is matching the barcode sequence of the unknown sample with the barcode library (known as sequence alignment) for its identification. For barcoding, Cytochrome c oxidase subunit I (COI) mitochondrial gene targeted can be amplified by universal primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3) /HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994, Hebert et al. 2003). The amplification reactions with composition of PCR mix [10X Taq buffer-5µl ($Mgcl_2$-15mM), dNTP's mix-1µl (2.5mM each), Taq DNA polymerase (1unit/ul)- 1µl (1unit), LCO1490-2µl (20pm), HCO2198-2µl (20pm), Template-5µl, NFW to make final volume-50µl] for amplification of COI of flies in 0.2ml PCR tubes using a programmable thermal cycler with cycling conditions [Initial denaturation-95^{o}C 5min, denaturation-95^{o}C 1min, annealing- 45^{o}C 1min, extension-72^{o}C 1min, final extension-72^{o}C 10min, stop reaction-4^{o}C 5min, 30 cycles] (Hebert et al. 2003). Later PCR products should be sequenced and the sequences then checked for homology by using the available bioinformatics tool BLAST (Basic local alignment search tool) from NCBI (National Centre for Biotechnology Information) server. Sequences submitted to GenBank and accession numbers allotted and then barcode generated using BoldSystem.

It was Paul Hebert who established that the mitochondrial gene cytochrome *c* oxidase I (COI) could serve as the core of a global bioidentification system for animals, where he revolutionised how every living thing on earth can be barcoded and identified, therefore he is regarded as the father of DNA barcoding. He showed that COI gene of animals and insects can be amplified by PCR, which yield a specific amplicon of 658bp. Various authors used COI barcoding by using universal primers by amplifying 658bp length of gene for identification of various medical and veterinary important insects such as Nelson (2008) used COI barcode to identify morphologically identified *Chrysomya* species (*C. flavifrons, C. latifrons, C. megacephala, C. nigripes, C. rufifacies, C. safranea, C. semimetallica, C. varipes*and *C. incisuralis*) from East Coast of Australia. Boehm *et al*. (2012) used it for identifying various flies belonging to Calliphoridae and Muscidae in Germany. Bhaskaran and Sebastian (2015) used the method for identifying *Lucilia sericata* from India. Nzelu et al. (2015) used it to identify sand flies in Peru. Arnaldos et al. (2015) used COI barcoding for identification of *Sarcophaga tibialis* and *Sarcophaga bullata* from Spain. Meiklejohn et al. (2013) for identification of immature life stages of a forensically important fleshfly (Diptera: Sarcophagidae). Archana et al. (2014) confirmed the identity of *C. imicola, C. oxystoma, C. peregrinus, C. anophelis, C. palpifer, C. huffi, C. innoxius* and *C. circumscriptus.* Gutierrez et al. (2014) identified sand fly species (Diptera, Psychodidae, Phlebotominae) in Colombia. Rolo et al. (2013) used it for identifying flies belonging to Calliphoridae and Muscidae in Iberian Peninsula. Pinto et al. (2015) identified neotropical sand flies (Diptera, Psychodidae, Phlebotominae) in Brazil. The 658 bp of COI sequence of *M. domestica, C. megacephala, H.capensis, H.illucens* and *S. ruficornis* after BLAST analysis confirmed the species which were identified based on morphology in Bangalore districts of Karnataka (Archana et al. 2016). Harrup et al. (2016) used DNA barcoding for identification of *Culicoides* in Southern India. Universal primers for 16S, 18S, and COI of ticks were designed and demonstratedthat DNA barcoding system could successfully identify Ixodida adults, nymph and larvae (Lv et al., 2013). Morphological and molecularcharacterization of oriental cat flea infestingsmall ruminants by barcodingconfirmed the *C.orientis* forms separate species because it forms a separatecluster to the subspecies of *C. felis* and also clustered as sisterspecies with *C. canis*(Ashwini et al., 2017).

The integration of various types of data such as morphological, ecological, physiological and molecular data including DNA barcoding will improve species discovery and description

process. It is evident that the molecular work on insects is insufficient in veterinary and medical fields hence it is a potential area for further work.

Reference:

1. Alam M S, Kato H, Fukushige M, WagatsumaY, Itoh M. (2012) Application of RFLP-PCR-based identification for sand fly surveillance in an area endemic for Kala-Azar in Mymensingh, Bangladesh.J Parasitol Res Article ID 467821, 4 p
2. Archana M, D'Souza P.E., Jalali S.K., Renukaprasad C, Ojha R. (2014) DNA barcoding of commonly prevalent *Culicoides* midges in South India. Indian J Anim Sci 85(1):37-39
3. Archana M, D'Souza P.E., Ojha R, Jalali SK, (2015) DNA barcoding of flies commonly prevalent in poultry farms of Bengaluru District. J Entomol Zool Stud 4(4): 228-233
4. Arnaldos MI, Ruiz C, Torres B, Begoña I, García MD, Mora DG, Serrano J (2015) DNA barcoding of two forensically important fleshly species (Diptera: Sarcophagidae) from spain and notes on barcoding success within genus *Sarcophaga meigen*, 1826. Ciencia Forense. 12:73-90
5. Ashwini MS, Puttalakshmamma GC, Mamatha GS, Rakshith Ojha, Chandranaik BM, Thimmareddy PM, Placid E D'souza, Jalali SK and Venkatshan T (2017) Studies on morphology and molecular characterization of oriental cat flea infesting small ruminants by barcoding. J Entomol Zool Stud 2017; 5(4): 301-305
6. Audtho M, Tassanakajon A, Boonsaeng V, Tpiankijagum S, Panyim S (1995) Simple nonradioactive DNA hybridization method for identification of sibling species of *Anopheles dirus* (Diptera: Culicidae) complex. J Med Entomol 32 (2): 107-111
7. Balczun C, Vorsprach B, Meiser C K, Schaub G A (2009) Changes of the abundance of *Culicoides obsoletuss.s*. and*Culicoides scoticus* in Southwest Germany identified by a PCR-based differentiation. Parasitol Res **1**05:345–349
8. Ball SL, Armstrong KF (2006) DNA barcodes for insect pest identification: a test case with tussock moths (Lepidoptera: Lymantriidae). Canadian J Res 36: 337–350
9. Bass C, Williamsonm S, Wilding C S, Donnelly M J, Field L M, (2007) Identification of the main malaria vectors in the *Anopheles gambiae* species complex using a TaqMan real-time PCR assay. Malar J 6:23 doi:10.1186/1475-2875-6-155

10. Bensasson D, Zhang D, Hartl DL, Hewitt GM (2001) Mitochondrial pseudogenes: evolutions misplaced witnesses. Trends Ecol Evol 16(6):314-321
11. Bernstein JA, Frank LA, Kania SA (2014) PCR amplification and DNA sequence identification of an unusual morphological form of *Demodex cati* in a cat. Vet Dermatol. 1-4
12. Bhaskaran PKP, Sebastian CD, (2015) Molecular barcoding of green bottle fly, *Lucilia sericata* (Diptera: Calliphoridae) using COI gene sequences. J Entomol Zool Stud 3(1):10-12
13. Boehm P, Amendt J, Zehner R, (2012) The use of COI barcodes for molecular identification of forensically important fly species in Germany. Parasitol Res 110:2325- 2332
14. Cetre-Sossah C, Mathieu B, Setier-rio M L, Grillet C, Baldet T, Dele´colle J C, Albina E (2008) Development and evaluation of a real-time quantitative PCR assay for *Culicoides imicola*, one of the main vectors of bluetongue (BT) and African horse sickness (AHS) in Africa and Europe. Res Vet Sci 85:372–382
15. Chambers EW, Meece JK, Mcgowan JA, Lovin DD, Hemme RR, (2011) Evaluation of a PCR-RFLP- ITS2 assay for discrimination of *Anopheles* species in northern and western Colombia. Acta Trop 118(2): 128–135
16. Cienfuegos AV, Rosero DA, Naranjo N, Lukhart JE, Correa MM (2011) Evaluation of a PCR-RFLP-ITS2 assay for discrimination of *Anopheles* species in northern and western Colombia. Acta Trop118(2)-128-135
17. Deblauw I, Dewitt JC, Dedeke G, Dedek ER, Madde R, Vaner S, Hoz FA, Lathouwer F, Geyse D (2011). A new tool for the molecular identification of *Culicoides* species of the Obsoletus group: the glass slide microarray approach. Med VetEnt doi: 10.1111/j.1365-2915.2011.00979
18. Folmer O M, Black W, Hoeh R, Lutz, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mole Mar Biol Biotechn 3(5): 294-299
19. Gutierrez MLC, Rafael J, Vivero IND, Lez V, Charles H, Porter US (2014) DNA Barcoding for the identification of sand fly species (Diptera, Psychodidae, Phlebotominae) in Colombia. PLoS ONE 9(1):1-9
20. Harrup LE, Laban S, Purse BV, Reddy YK, Reddy YN, Byregowda SM, Kumar N, Purushotham KM, Kowalli S, Prasad M, Prasad G, Bettis AA, Keyser RD, Logan J, Garros

C, Gopurenko D, Bellis G, Labuschagne K, Mathieu B, Carpenter S (2016) DNA barcoding and surveillance sampling strategies for Culicoides biting midges (Diptera: Ceratopogonidae) in southern India. Parasit Vectors 9:461

21. Hebert PDN, Cywinska A, Ball SL, Dewaard JR (2003) Biological identifications through DNA barcodes. Proc R Soc London B 270:313-321
22. Higa Y, Toma T, Tsunda Y, Miyagi I (2010) A Multiplex PCR-based molecular identification of five morphologically related, medically important subgenus Stegomyia mosquitoes from the genus *Aedes* (Diptera: Culicidae) found in Ryukyv Japan. Jpn J InfectDis 63: 312-316
23. Jalali SK, Ojha R, Venkatesan T (2015) DNA barcoding for identification of agriculturally important insects. Chakravarthy AK (ed), New Horizons in Insect Science: Towards Sustainable Pest Management. 13-23, NBAIR
24. Kanojia PC, Paingankar MS, Patil AA, Gokhale MD, Deobagkar DN (2009) Morphometric and allozyme variation in *Culex tritaeniorhynchus* mosquito populations Indian J Insect Sci Vol. 10 Article 138.
25. Kiehl E, Walldorf V, Klimpel S, Quraishy S A, Mehlhorn H (2009) The European vectors of Bluetongue virus: are there species complexes, single species or races in *Culicoides obsoletus* and *C. pulicaris* detectable by sequencing ITS-1, ITS-2and 18S-rDNA. Parasitol Res 105:331–336
26. Kim DW, Yoo WG, Park HC, Yoo HS, Kang DW, Jin SD, Min HK, Paek WK, Lim J (2012) DNA barcoding of fish, insects, and shellfish in Korea. Genomics Inform 10(3):206-211
27. Lall GK, Darby AC, Nystedt B, Macleod ET, Bishop RP, Welburn SC (2010)0Amplified fragment length polymorphism (AFLP) analysis of closely related wild and captive tsetse fly (*Glossina morsitans morsitans*) populations Parasit Vectors 3:47
28. Loxdale H D, Lushai G (1998) Molecular markers in entomology. Bull Ent Res 88:577-600
29. Lv J, Wu1 S, Zhang Y, Zhang T, Feng C, Jia G, and Lin X (2013) Development of a DNA barcoding system for the Ixodida (Acari: Ixodida). Mitochondrial DNA. Early Online: 1–8
30. Mandal SD, Chhakchhuak L, Gurusubramanian G, Kumar NS(2014) Mitochondrial markers for identification and phylogenetic studies in insects – A Review. DNA Barcodes 2: 1–9

31. Mazzanti M, Alessandrini F, Tagliabracci A, Wells JD, Campobasso CP (2010) DNA Degradation and genetic analysis of empty puparia: Genetic identification limits in forensic entomology. Forensic Sci Int195:99-102
32. Meiklejohn KA (2012) Taxonomy and systematic of Australian Sarcophagas. l. (Diptera: Sarcophagidae). University of Wollongong thesis collections. University of Wollongong
33. Monaco F, Benedetto L, MarcelloV D, Lelli R, Goffredo M (2010) Development and Preliminary evaluation of a real time polymerised chain reaction for the identification of C. *obseletussensostrictu, C. scoticus, C. montanus* in *Obseletus* complex in Italy. Vet Ital 46(2): 215-220
34. Nelson LA (2008) Molecular identification and thermal attributes of forensically important blowflies (Diptera: Calliphoridae: Chrysomya). University of Wollongong thesis collections. University of Wollongong
35. Nolan DV, Carpenter S, Barber J, Mellorp S, Dallas JF, Mordue AJ, Piertney SB (2007) Rapid diagnostic PCR assays for members of the *Culicoides obsoletus and Culicoides pulicaris* species complexes, implicated vectors of bluetongue virus in Europe. Vet Mic124: 82–94
36. Nzelu CO, Cáceres AG, Jiménez MJA, Rosas MFL, Trujillano HHY, Caipo DVL (2015) DNA barcoding for identification of sand fly species (Diptera: Psychodidae) from leishmaniasis-endemic areas of Peru. Acta Trop 145:45-51
37. Pinto Ide S, Chagas BD, Rodrigues AA, Ferreira AL, Rezende HR, Bruno RV, Falqueto A, Andrade-Filho JD, Galati EA, Shimabukuro PH, Brazil RP, Peixoto AA (2015) DNA Barcoding of Neotropical Sand Flies (Diptera, Psychodidae, Phlebotominae): Species Identification and Discovery within Brazil. PLoS one 1-18
38. Rivera J, Currie DC (2009) Identification of Nearctic black flies using DNA barcodes (Diptera: Simuliidae). Mol Ecol Res 9:224-236
39. Rolo EA, Oliveira AR, Dourado CG, Farinha A, Rebelo MT, Dias D (2013) Identification of sarcosaprophagous Diptera species through DNA barcoding in wildlife forensics. Forensic Sci Int 228:160-164
40. Ronaghi H, Nabian S, Ebrahimzadeh E, Biranvand F, Shayan P (2015) Molecular characterization of Rhipicephalus (Boophilus) annulatus from Iran by sequences of

cytochrome c oxidase subunit I (COI) and the second internal transcribed spacer (ITS2). IJVM (2015), 9(2):117-123

41. Saccone C, DeCarla G, Gissi C, Pesole G, Reynes A (1999) Evolutionary genomics in the Metazoa: the mitochondrial DNA as a model system. Gene 238: 195–210
42. SilvaM H, Nascimento M, LeonardoFS, RebeloJM, Pereira SR (2011) Genetic Diff erentiation in Natural Populations of *Lutzomyia longipalpis*(Lutz & Neiva) (Diptera: Psychodidae) with Different Phenotypic Spot Patterns on Tergites in Males. Neotrop Entomol 40(4): 501-506
43. Singh D Achint R (2017) Molecular identification of some Indian Muscid flies (Diptera: Muscidae) based on mitochondrial gene COII. Inter J Zool Stud. 2 (6):101-105
44. Sossah CC, Baldet T, Delécolle JC, Mathieu B, Perrin A, Grillet C, Albina E (2004) Molecular detection of *Culicoides* spp. and *Culicoides imicola*, the principal vector of bluetongue (BT) and African horse sickness (AHS)in Africa and Europe.Vet Res35: 325-337
45. Sperling FAH, Anderson GS, Hickey DA (1994) A DNA-based approach to the identification of insect species used for postmortem interval estimation. J Forensic Sci39:418-27
46. Stephan A, Clausen PH, Bauer B, Steuber S (2009) PCR identification of *Culicoides dewulfi* midges (Diptera:Ceratopogonidae), potential vectors of bluetongue in Germany. Parasitol Res 105:367-371
47. Walker ED, Thibault AR, Thelen AP, Bullard BA, Huang J, Odiere MR, Bayoh NM, Wilkins EE, Vulule JM (2007) Identification of field caught *Anopheles gambiae*s.s. and*Anopheles arabiensis*by TaqMan single nucleotide polymorphism genotyping. Malar J6:23 doi:10.1186/1475-2875-6-23
48. Wirth WW Marston N (1967) A method for mounting small insects on microscope slides in canada balsam. Ann. Entomol. Soc. Am. 61: 783–784
49. Wirth WW, Hubert AA (1989) The*Culicoides* of Southeast Asia Diptera (Ceratopogonidae). Mem Amer Ent Inst no 44

Histopathological approaches for detection of parasitic diseases

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Introduction: Histopathology is a part of pathology and deals with the study of disease animals. Histopathology represents a science on structure and function of tissues in ill or diseased animals. Histopathological analysis plays an important role in the diagnosis of human and animal diseases of different etiologies such as infectious, neoplastic, parasitic, deficiency diseases and of intoxications. In many cases, parasitic diseases are not properly recognized. A cursory diagnosis often leads to prolonged or even ineffective treatment. Histopathological examination of organs or tissues facilitates a thorough and accurate diagnosis(Zenon et al., 2014). In parasitic diseases diagnosis, histopathological examination is not been considered as diagnostic tool but gaining importance in recent years. Very often, histopathological examination allows to identify the parasite species involved, the area of pathological lesions, possible complications of bacterial or viral origin, and the outlook of treatment.

Histopathological changes caused by flatworms: Liver fluke disease of sheep and cattle, and occasionally other species, most commonly is due to the juvenile *Fasciola hepatica* fluke injures the hepatic parenchyma when moving to the bile ducts. Migration of immature flukes through the liver produces hemorrhagic tracts of necrotic liver parenchyma. These tracts are grossly visible and in acute infestation, are dark red, but with time become paler than the surrounding parenchyma. Repair process is often by fibrosis. The *Schistosoma haematobium* fluke eggs use their spine to penetrate the wall of the urinary bladder and enter the external environment with the host's urine. The *Schistosoma mansoni* fluke eggs break through the wall of the colon into the gastrointestinal tract and in doing so damage the lamina and mucosa of the large intestine. The masses of eggs become surrounded by inflamed areas infiltrated by leucocytes, particularly eosinophils, in internal organs such as the liver. The larval form of *Taenia pisiformis* burrows tunnels through the hepatic parenchyma of rabbits, damaging their liver parenchyma and inducing the formation of a peripheral inflammatory infiltrate composed of eosinophiles and giant cells. In chronic cases, the damaged liver parenchyma produces fibrotic scars. The larvae of *Echinococcus multilocularis s. alveolaris* are often located in the liver, lungs or brain. The larval

forms are surrounded by inflammatory infiltration with numerous eosinophils, necrosis and calcification (Taylor et al., 2007). The diagnosis is confirmed by histological detection of Echinococcus larval hooks.

Histopathological changes caused by roundworms: The wild boar lungs examined were focally congested, dense in consistence; the altered pulmonary parenchyma in a water test was airless. Parasitic changes in the lungs were present in all the wild boars examined. Dark red lobuli were surrounded by centrilobular emphysema light pink in colour. The pulmonary pleura in place in the centrilobular emphysema was slightly elevated. The changes affected mainly the lower parts of the main lobes of both lungs. The enlarged light bronchioles and bronchi revealed the presence of numerous nematodes in the bronchiolitis mucus. Abundant nematodes in the bronchioli (over 20 nematodes) cause the extending the cross-section of broncholi. Abundant nematodes in the bronchioli (over 20 nematodes) cause the dilatation of the bronchioli light. The presence of adult nematodes in the bronchial tree, their movements and their excreta as well as the antigens secreted cause inflammation of the bronchial tree, and then inflammation in the tissue and alveoli causing bronchopneumonia. Larvae migrating through the bronchial alveoli also damage alveoli the walls and cause lung tissue inflammation. The larval nematode migration in the pulmonary parenchyma to the bronchi and bronchioles caused changes and trauma leading to the development of inflammation expressed by infiltration of inflammatory cells with numerous eosinophils. The mechanical damage healed through scarring, i.e., the formation of the connective scar tissue involved in the repair process leading to extensive lung fibrosis. In the lung parenchyma was also reported small necrotic foci, that have been calcified. Mobility of nematodes and antigens secreted, their excreta, and the presence of eggs resulted in chronic bronchitis and bronchiolitis with eosinophilic cells(Zenon et al., 2014). Migrations of larval Metastrongylus nematodes in the lung parenchyma caused catarrhal pneumonia, the secondary purulent infection being caused by staphylococci (*Staphylococcus spp*.).

Respiratory tract infections are caused by parasites, bacteria or viruses, and mixed respiratory tract infections are very common. The interaction of pathogens exacerbates the disease, whereby pathological changes are more complicated (Zenon et al., 2014). Numerous authors confirmed the usefulness of histopathological examination in evaluation of the disease process. The concurrent porcine circovirus type 2 may trigger metastrongylosis, which may

subsequently result in severe, and sometimes fatal, pulmonary disease (Marruchella et al., 2012). Histopathological analysis helped to fully determine the causes of the disease in the animals affected.

Histopathological changes caused by arthropods: In dogs, burrowing mites *(Sarcoptes scabiei v. canis)* parasitize in the deep layers of the epidermis (stratum granulosum, s. spinosum). The changes are located on the head, rump and at the base of the tail. At the beginning of the invasion, the lesions occur as local, limited skin exfoliation which next creates scaly raids. As the disease progresses, the skin is reddened and thickened, and corneous epidermis forms papule.Demodex canis occupies the hair follicles and sebaceous glands and causes generation of small nodules with thinned hair, and excessive skin desquamation. Initially, the changes appear on the head, then small lesions form larger, clearly demarcated, red scalp patches extending to the neck, forelegs and the rest of the body. The disease is chronic in nature.

The presence of *Oestrus ovis* larvae causes inflammation of the mucous membrane of the nasal cavity and sinus. Occasionally, the larvae may penetrate the skull bones and enter the cerebral cavity; clinical signs are similar to those caused by *Taenia multiceps* called the false gid. Changes in the mucous membrane of frontal sinus include catarrh, inflammatory cells infiltration and squamous metaplasia. The points of attachment of L2 and L3 larvae of *Gasterophilus intestinalis* in the glandular and non-glandular stomach of horses are marked by wastages on both the corneous layer and the squamous mucosa, accompanied by its focal hyperkeratosis. The necrosis focus showed bacterial colonies and infiltration of eosinophils and neutrophils. The bottom of the wastage in the proper epidermal layer of the mucous membrane featured diffuse infiltration of inflammatory cells.

Histopathological changes caused by Protozoan parasites: Trypanosomosis has a wide range of hosts and is pathogenic to most of the domestic and laboratory animals. The parasite utilizes glucose and oxygen for its growth and multiplication resulting in depletion of these metabolites leading to degenerative changes in the host. Further changes develop in the organs either due to cellular damage caused by toxicants released by the parasite or due to immunological reactions. In an experimental study conducted in Rats showed lesions in liver, kidney, spleen and trypanasomes attached to the heart endocardium on histological examinations (Krishnamoorthy et al., 2016). The decrease in serum glucose and increase in creatinine level was observed on

serum biochemistry. In our immunohistochemistry study in rats infected with Trypanosoma evansi, we found that the trypanosome antigens was detected in spleen tissue section compare to other organs. This may be due to site of destruction of trypanosomes was spleen and could be detected by immunohistochemistry. Emaciated animal markedly, spleen and lymph glands are enlarged and show hyperplasia of follicles. There may be multiplication of the macrophages in sinuses and infiltration by lymphocytes and plasma cells. Later organization occurs with suppression of glandular function. Congestion of bone marrow and of the gastrointestinal mucosa may be present. Subcutis shows gelatinous infiltration with petechiae on serous mucous membranes. Serous exudate into pericardial cavity and peritoneum is seen. Ulceration of tongue and gastric mucosa was observed (Bal et al., 2012).

Death in Trypansomiasis may be attributable to

1. Endotoxin that may be released by the lysis of parasites

2. Asphyxia due to increased blood lactic acid

3. Hypoglycemia

4. Toxaemia that develops due to dysfunction of liver and the cause of hepatic dysfunction is destruction of large quantities of glucose.

5. Erythrocyte production is inhibited by toxins

5. Trypanosomes may liberate proteolytic ferments, which digesting proteins may liberate toxic products.

Theileriosis are a group of tickborne diseases caused by *Theileria spp*. A large number of *Theileria spp* are found in domestic and wild animals in tick-infested areas of the Old World. The most important species affecting cattle are *T. parva* and *T. annulata*, which cause widespread death in tropical and subtropical areas of the Old World. *T. lestoquardi*, *T. lowenshuni* and *T. uilenbergi* are important causes of mortality in sheep.Main post-mortem findings were oedema and enlargement of, and haemorrhages in lymph glands and spleen, haemorrhages in subcutis and on most of the serous and mucous membranes on endocardium, pericardium and epicardium, and ulcers in abomasum which rarely extended to intestine,

oesophagus, tongue and gums. The microscopic lesions were characterized by hyperplasia of lymphoid cells at the haemopoietic centres in lymph glands and spleen followed by regression and degeneration of the germinal centres. Infiltration by lymphocytes and macrophages was observed in intermysial (heart) and periportal (liver) areas in interstitial spaces in kidneys. *T. annulata* parasitizes lymphoid cells. It provides stimulus for accelerated production of the cells which results in hyperplasia of the lymphoid tissue. Increased activity of the reticular tissue is a protective phenomenon in animals (Sastry, 1983).

Bovine babesiosis is also the most economically important disease because of direct loss on production.Gross pathology seen in cases of babesiosis can be highly variable:

- Varying degrees of congestion, pallor or jaundice
- Blood is usually watery and urine is red
- Sub-serosal haemorrhages are common, particularly on the heart and intestines
- Spleen is enlarged with a soft red pulp
- Liver is enlarged and brown or yellow, with the gall bladder filled with thick, granular bile
- Lesions observed are those most often associated with an intravascular haemolytic condition
- Pale or icteric mucous membranes; blood may appear thin and watery
- Subcutaneous tissues, abdominal fat and omentum may appear icteric
- Swollen liver with an orange-brown or paler coloration; enlarged gall bladder containing thick, granular bile
- Enlarged, dark, friable spleen
- Kidneys appear darker than normal with possible petechial haemorrhages
- Bladder may contain dark red or brown-colored urine
- Possible oedema of lungs

In ruminants, tick-borne fever occurs in domesticated ruminants, particularly sheep and cattle. It has also been documented in goats, deer and reindeer. In sheep, this disease is mainly seen in young lambs born in tick-infested areas, and in newly introduced older sheep. Tick pyemia, caused by *Staphylococcus* spp., is the most frequent and severe complication in young

lambs. This illness is characterized by severe lameness, debility and paralysis, and many lambs die. Pasteurellosis and septicemic listeriosis are also common complications. In Canines, gross lesions are usually nonspecific in acute cases, but typically include enlargement of the spleen. Hemorrhagic lesions in severe canine ehrlichiosis can affect numerous organs including the gastrointestinal tract, heart, bladder, lungs, subcutaneous tissues and eyes. The lymph nodes, particularly the mesenteric nodes, may be enlarged, with red and brown discoloration on cut surface. There may be edema in the legs, as well as ascites and hydro pericardium. In chronic canine ehrlichiosis, splenomegaly and nonspecific lesions may be accompanied by widespread haemorrhages and microscopically by mononuclear cell infiltration into the perivascular area in multiple organs. The characteristic lesions of equine granulocytic anaplasmosis are petechiae, ecchymoses and edema in the subcutaneous tissues and fascia, mainly in the legs. Interstitial pneumonitis has been reported in some animals (CFSPH report, 2013).

A male Wistar albino rat about 22 weeks of age showed the presence of numerous cysts in liver. Gross examination of liver showed white to yellowish cyst of 0.2 to 0.6 cm diameter, on incision, fluid or purulent exudates and larvae was seen. Histopathological examination revealed well-developed cyst wall with inner layer composed of cytokeratin along with thick connective layers mainly of fibrous tissue. The hepatocytes were damaged with severe infiltration of eosinophils and plasma cells surrounding the cyst. The larva showed the presence of an outer acellular eosinophilic cuticular layer and underlying subtentacular layer along with scolex containing hooks and suckers. Thus, based on the gross and histopathological examination, the accidental finding of cysts was confirmed to be Cysticercosis infection in Wistar albino rat (Krishnamoorthy et al., 2017).

The histopathological analysis of the large intestines and livers of turkeys infected revealed numerous oval or circular protozoan parasites with granular endoplasm, representing *Histomonas meleagridis*. Histopathological examination of the large intestine and liver of reptiles revealed the presence of *Entamoeba invadens*. Theseprotozoan parasites are oval in shape, their nuclei and karyosomes being well-stained. The study showed cellular and topographic changes of the liver and caecum in turkeys infected with *H. meleagridis*, thus confirming the pattern of the hepatic and caecal infections by *H. meleagridis* in birds. The cross-sections of the intestinal wall of pigs infected with *Balantidium coli* revealed round or oval protozoan parasites with bean-shaped macronuclei. The protozoans surrounded the inflammatory

infiltrate composed of lymphocytes, histiocytes, and eosinophils. In coccidiosisaffected rabbits, the schizogony and gametogony damaged the biliary epithelium andproduced clinical symptoms. The coccidia schizogony and gamogony in some rabbit species occur in the small or large intestine producing histologically detectable inflammatory changes. Thin gamonts of *Eimeria necatrix, E. acervulina, E. precox, E. mivati, E. maxima, E. brunetti* and *E. tenella* were visible within the epithelial cells of the small and large intestinal mucosa of chickens. The cross-sections of the intermediate host's skeletal muscles revealed many zoites (*Sarcocystis spp*.) within sarcocysts (Mehlhorn, 2001, Taylor et al., 2007).

Conclusions

Histopathological examination is useful for a number of reasons: a) to detect parasites; b) to reveal the area of tissue damage caused by migrating larval forms and mature parasites; c) to apply appropriate treatment; d) to explain why certain treatments are not effective during a parasite invasion (parasites damage parenchymal organs causing permanentorgan dysfunction and inducing formation of fibrotic scars). Histopathological examination provides insights into interactions between pathogens and their impacts on the host organism, particularly in multi-etiological infections. Hence, it is advised to consider histopathological examination tissues of animals affected with parasitic diseases for accurate disease diagnosis.

References

1. Bal MS, Singla LD, Kumar H, Vasudev A, Gupta K, Joyal PD. 2012. Pathological studies on experimental *Trypanosoma evansi* infection in Swiss albino mice. *Journal of Parasitic Diseases*, 36(2): 260-264
2. CFSPH report. 2013. Erhlichiosis and Anaplasomosis: zoonotic species. The Centre for Food Security and Public Health, Iowa State University, USA.
3. Krishnamoorthy P, Sengupta PP, Balachandran C and Roy P. 2017. Cysticercosis in a Wistar Albino Rat. *Research & Reviews: Journal of Veterinary Science and Technology*, 6(2): 23-25.
4. Krishnamoorthy P, Sengupta PP, Das S, Ligi M, Shome BR, Rahman H. 2016. Cytokine gene expression and pathology in mice experimentally infected with different isolates of *Trypanosoma evansi.Experimental Parasitology*, 170: 168-176.

5. Marruchella G, Paoletti B, Speranza R, Di Guardo G. 2012. Fatal bronchopneumonia in a Metastrongylus elongatus and Porcine circovirus type 2 coinfected pig. *Research in Veterinary Science*, 93: 310-312.
6. Mehlhorn H. 2001. *Encyclopedic Reference of Parasitology (Biology, Structure, Function)*. SpringerVerlag Berlin, Heidelberg, New York.
7. Sastry GA. 1983. *Veterinary Pathology*, CBS publishers and distributors, New Delhi, India. pp:685-692.
8. Taylor MA, Coop RL, Wall RL. 2007. Veterinary Parasitology, 3rd ed., Blackwell Publishing Ltd, Oxford.
9. Zenon S, Jerzy R, Magdalena K. 2014. Histopathological diagnosis in parasitic diseases. *Annals in Parasitology,* 60(2): 127-131.

Genome Sequencing methods and its application

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DNA sequencing is one of the most important platforms for the study of biological systems today. The term DNA sequencing gencompasses biochemical methods for determining the order of the nucleotide bases, adenine, guanine, cytosine, and thymine, in a DNA oligonucleotide. The sequence of DNA constitutes the heritable genetic information in nuclei, plasmids, mitochondria, and chloroplasts that forms the basis for the developmental programs of all living organisms. Determining the DNA sequence is therefore useful in basic research studying fundamental biological processes, as well as in applied fields such as diagnostic or forensic research. The advent of DNA sequencing has significantly accelerated biological research and discovery. The rapid speed of sequencing attainable with modem DNA sequencing technology has been instrumental in the large-scale sequencing of the human genome. Genome related projects, often by scientific collaboration across continents, have generated the complete DNA sequences of many animal, plant, and microbial genomes.

For thirty years, a large proportion of DNA sequencing has been carried out with the chain-termination method, developed by Frederick Sanger and co-workers in 1975. Prior to the development of rapid DNA sequencing methods in the early 1970s by Sanger in England and Gilbert et al. at Harvard, a number of laborious methods were used. For instance, in 1973 Gilbert and Maxam reported the sequence of 24 base pairs using a method known as wandering-spot analysis. In 1976-1977, Allan Maxam and Waiter Gilbert developed a DNA sequencing method based on chemical modification of DNA and subsequent cleavage at specific bases. Maxam-Gilbert sequencing (Chemical sequencing) rapidly became more popular, since purified DNA could be used directly, while the initial Sanger method required that each read start be cloned for production of single-stranded DNA. However, with the development and improvement of the chain-termination method, this method has fallen out of favour due to its technical complexity, extensive use of hazardous chemicals, and difficulties with scale-up. In addition, unlike the chain-termination method, chemicals used in the Maxam-Gilbert method cannot easily be customized for use in a standard molecular biology kit.

This method requires radioactive labelling at one end and purification of the DNA fragment to be sequenced. Chemical treatment generates breaks at a small proportion of one or two of the four nucleotide bases in each of four reactions (G, A+G, C, C+T). Thus, a series of labeled fragments is generated, from the radio labeled end to the first 'cut' site in each molecule. The fragments are then size-separated by gel electrophoresis, with the four reactions arranged side by side. To visualize the fragments generated in each reaction, the gel is exposed to X-ray film for autoradiography, yielding an image of a series of dark 'bands' corresponding to the radiolabeled DNA fragments, from which the sequence may be inferred. Chemical and the plus-minus method of Sanger and Coulson were orders of magnitude faster than previous methods, the chain-terminator method developed by Sanger was even more efficient, and rapidly became the method of choice. The Maxam-Gilbert technique requires the use of highly toxic chemicals, and large amounts of radio labeled DNA, whereas the chain-terminator method uses fewer toxic chemicals and lower amounts of radioactivity. The key principle of the Sanger method was the use of dideoxynucleotides triphosphates (ddNTPs) as DNA chain terminators.

The classical chain-termination or Sanger method requires a single-stranded DNA template, a DNA primer, a DNA polymerase, radioactively or fluorescently labeled nucleotides, and modified nucleotides that terminate DNA strand elongation. The DNA sample is divided into four separate sequencing reactions, containing the four standard deoxynucleotides (dATP, dGTP, dCTP and dTTP) and the DNA polymerase. To each reaction is added only one of the four dideoxynucleotides (ddATP, ddGTP, ddCTP, or ddTTP). These dideoxynucleotides are the chain- terminating nucleotides, lacking a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides during DNA strand elongation. Incorporation of a dideoxynucleotide into the nascent (elongating) DNA strand therefore terminates DNA strand extension, resulting in various DNA fragments of varying length. The dideoxynucleotides are added at lower concentration than the standard deoxynucleotides to allow strand elongation sufficient for sequence analysis.

The newly synthesized and labeled DNA fragments are heat denatured, and separated by size (with a resolution of just one nucleotide) by gel electrophoresis on a denaturing polyacrylamide-urea gel. Each of the four DNA synthesis reactions is run in one of four individual lanes (lanes A, T, G, C); the DNA bands are then visualized by autoradiography or UV light, and the DNA sequence can be directly read off the X-ray film or gel image. The

relative positions of the different bands among the four lanes are then used to read (from bottom to top) the DNA sequence as indicated. In one method, the DNA fragments are tagged with nuc1eotides containing radioactive phosphorus for radiolabelling. Alternatively, a primer labeled at the 5' end with a fluorescent dye is used for the tagging. Four separate reactions are still required, but DNA fragments with dye labels can be read using an optical system, facilitating faster and more economical analysis and automation. This approach is known as 'dye-primer sequencing'. The later development by L Hood and co-workers of fluorescently labeled ddNTPs and primers set the stage for automated, high-throughput DNA sequencing.

The major advantage of this Dye-terminator sequencing method is that the sequencing can be performed in a single reaction, rather than four reactions as in the labelled-primer method. In this method, each of the four dideoxynucleotide chain terminators is labelled with a different fluorescent dye, each fluorescing at a different wavelength. This method is attractive because of its greater expediency and speed and is now the mainstay in automated sequencing with computer-controlled sequence analyzers. Its potential limitations include dye effects due to differences in the incorporation of the dye-labelled chain terminators into the DNA fragment, resulting in unequal peak heights and shapes in the electronic DNA sequence trace chromatogram after capillary electrophoresis. This problem has largely been overcome with the introduction of new DNA polymerase enzyme systems and dyes that minimize incorporation variability. This method, along with automated high-throughput DNA sequence analyzers, is now being used for the vast majority of sequencing projects, as it is both easier to perform and lower in cost than most previous sequencing methods.

Pyrosequencing is a method of DNA sequencing (determining the order of nuc1eotides in DNA) based on the "sequencing by synthesis". This principle developed initially by Pal Nyren and co-workers during a period from 1985 to the late 1990s, then further by Biotage. The first major improvement was substitution of dATPcx.S for dATP in the polymerization reaction, which enabled the pyrosequencing reaction to be performed in homogeneous phase in real time. This technique is a widely applicable, alternative technology for the detailed characterization of nucleic acids. The technique dispenses with the need for labeled primers, labeled nucleotides, and gel-electrophoresis. The fastest of all the sequencing methods. It can sequence 10 million bases/hour. It is a method primarily used for sequencing of short stretches of DNA, SNP (small genetic variations) detection and methylation analysis. Such analyses are crucial for biological

research, genetics and some medical and forensic applications. Pyrosequencing is fully automated, reliable and accurate, flexibility, parallel processing, and large numbers of samples can be analysed in a short time. These methods have been pursued to reduce costs relative to other automated sequencing methods.

The technique has been further developed by the company 454 into a technology known as 454 Pyrosequencing. To date this is the fastest sequencing method. However, a limitation of the method is that read lengths are currently somewhat shorter than those obtained by di-deoxy nucleotide based chain termination methods, which, makes the process of genome assembly more complicated, particularly for genomes which contain a large amount of repetitive DNA. Pyrosequencing is most commonly used for resequencing or sequencing of genomes for which the sequence of a close relative is already available. The templates for pyrosequencing can be made both by solid phase template preparation (Streptavidin coated magnetic beads) and enzymatic template preparation (Apyrase + Exonuclease). A single run can generate around 30Mb of sequence, with an estimated cost per kilobase of raw sequence that is IO-fold less than conventional sequencing. The disadvantage of this technique is the short read length, which is currently 26bp per amplicon (13bp per tag). The colony method has now been taken by Applied Biosystems (Foster City, CA, USA). They have adapted the method so it is capable of 50bp reads and generating> 1Mb of sequence in a single run. The technology now named as SOLiD.

New generation sequence (NGS)

The quality and quantity of nucleic acids are important for NGS, as generally, an initial quality check helps find samples likely to produce low performing NGS libraries due to fragmentation or degradation. Further, confirming the size, concentration, and overall quality of prepared libraries helps optimize cluster generation during sequencing and aids in both cost and time savings while maximizing NGS data quality and output. Further, instruments that can accurately assess nucleic acid quality and quantity is also important to successful sequencing.

Many of the problems, and inherent errors, of DNA sequencing result from the fact that thousands or millions of amplified templates are assessed in a single-molecule sequencing reaction. It would be far better to read DNA in the same way as cells do; as single molecules. This method involves hybridizing target DNA to complimentary primers that are streptavidin-biotin bound to a silica surface. The primers are then extended by the addition of Cy3- and Cy5-labeled nucleotides; as each base is added, the incorporation is captured using a camera mounted

on a microscope. A limitation of this technology is that it generates short reads, which at the time of publication was 5bp; however, this technology has been taken up by Helicos Biosciences Corporation, Cambridge, MA, USA, who are reporting much longer reads. This method is highly parallel, and on a 25mm square it would be possible to sequence 12 million templates simultaneously, so, even with 5bp reads, each 'run' would generate 60 million bases of information.

Nanopore sequencing is other method of single-molecule sequencing that is in the very early stages of development involves 'reading' DNA as it is passed through a nanopore. This would not involve an enzymatic extension reaction of any kind but instead the physical properties of the molecule would be read as the bases wind through a tiny pore. In theory, this method would have no limit on read length and, hence, if the technical hurdles are overcome it could revolutionize how genome sequencing is achieved. The physicists used mathematical calculations and computer modeling of the motions and electrical fluctuations of DNA molecules to determine how to distinguish each of the four different bases (A, G, C, T) that constitute a strand of DNA. They based their calculations on a pore about a nanometer in diameter made from silicon nitride-a material that is easy to work with and commonly used in nanostructures=-surrounded by two pairs of tiny gold electrodes. The electrodes would record the electrical current perpendicular to the DNA strand as the DNA passed through the pore. Because each DNA base is structurally and chemically different, each base creates its own distinct electronic signature.

Modem automated DNA sequencing instruments (DNA sequencers) can sequence up to 384 fluorescently labelled samples in a single batch (run) and perform as many as 24 runs a day. However, automated DNA sequencers carry out only DNA size separation by capillary electrophoresis, detection and recording of dye fluorescence, and data output as fluorescent peak trace chromatograms. Sequencing reactions by thermocycling, clean up and re-suspension in a buffer solution before loading onto the sequencer are performed separately.

The field of DNA sequencing technology development has a rich and diverse history. However, the overwhelming majority of DNA sequence production to date has relied on some version of the Sanger biochemistry. In high-throughput production pipelines, DNA to be sequenced is prepared by one of two approaches: first, for shotgun *de novo* sequencing, randomly fragmented DNA is cloned into a high-copy-number plasmid, which is then used to transform *Escherichia coli;* or second, for targeted resequencing, PCR amplification is carried

out with primers that flank the target. With high-throughput shotgun Sanger sequencing, genomic DNA is fragmented, then cloned to a plasmid vector and used to transform *E. coli.* For each sequencing reaction, a single bacterial colony is picked and plasmid DNA isolated. Each cycle sequencing reaction takes place within a microliter- scale volume, generating a ladder of ddNTP-terminated, dye-labelled products, which are subjected to high-resolution electrophoretic separation within one of 96 or 384 capillaries in one run of a sequencing instrument. As fluorescently labelled fragments of discrete sizes pass a detector, the four-channel emission spectrum is used to generate a sequencing trace.

The shotgun method is the most practical for sequencing large genomes, but its assembly process is complex and potentially error-prone- particularly in the presence of repeating sequences. In shotgun sequencing with cyclic-array methods, common adaptors are ligated to fragmented genomic DNA, which is then subjected to one of several protocols that results in an array of millions of spatially immobilized PCR colonies or 'polonies. Each colony consists of many copies of a single shotgun library fragment. As all polonies are tethered to a planar array, a single microliter-scale reagent volume (e.g., for primer hybridization and then for enzymatic extension reactions) can be applied to manipulate all array features in parallel. Similarly, imaging-based detection of fluorescent labels incorporated with each extension can be used to acquire sequencing data on all features in parallel. Successive iterations of enzymatic interrogation and imaging are used to build up a contiguous sequencing read for each array feature

The 454 system was the first next-generation pyro sequencing platform available as a commercial product. In this approach, libraries may be constructed by any method that gives rise to a mixture of short, adaptor-flanked fragments. Clonal sequencing features are generated by emulsion PCR, with amplicons captured, to the surface of beads. After breaking the emulsion, beads are treated with denaturant to remove untethered strands, and then subjected to a hybridization-based enrichment for amplicon- bearing beads (that is, those that were present in an emulsion compartment supporting a productive PCR reaction). A sequencing primer is hybridized to the universal adaptor at the appropriate position and orientation, that is, immediately adjacent to the start of unknown sequence.

Illumina Genome Analyzer: Commonly referred to as 'the Solexa', this platform has its origins in work by Turcatti and colleagues and the merger of four companies-Solexa (Essex,

UK), Lynx Therapeutics (Hayward, CA, USA), Manteia Predictive Medicine (Coinsins, Switzerland) and Illumina. Libraries can be constructed by any method that gives rise to a mixture of adaptor- flanked fragments up to several hundred base-pairs (bp) in length. Amplified sequencing features are generated by bridge PCR. In this approach, both forward and reverse PCR primers are tethered to a solid substrate by a flexible linker, such that all amplicons arising from any single template molecule during the amplification remain immobilized and clustered to a single physical location on an array. AB SOLiD platform has its origins in the system described by J.S. and colleagues in 2005 and in work by McKernan and colleagues at Agencourt Personal Genomics (Beverly, MA, USA) (acquired by Applied Biosystems (Foster City, CA, USA) in 2006). Libraries may be constructed by any method that gives rise to a mixture of short, adaptor-flanked fragments, though much effort with this system has been put into protocols for mate-paired tag libraries with controllable and highly flexible distance distributions. Clonal sequencing features are generated by emulsion PCR, with amplicons captured to the surface of I-M paramagnetic beads. After breaking the emulsion, beads bearing amplification products are selectively recovered, and then immobilized to a solid planar substrate to generate a dense, disordered array. Sequencing by synthesis is driven by a DNA ligase, rather than a polymerase.

The Helicos sequencer, based on work by Quake's group, also relies on cyclic interrogation of a dense array of sequencing features. However, a unique aspect of this platform is that no clonal amplification is required. Instead, a highly sensitive fluorescence detection system is used to directly interrogate single DNA molecules via sequencing by synthesis. Template libraries, prepared by random fragmentation and poly- A tailing (no PCR amplification), are captured by hybridization to surface- tethered poly- T oligomers to yield a disordered array of primed single-molecule sequencing templates. At each cycle, DNA polymerase and a single species of fluorescently labeled nucleotide are added, resulting in template-dependent extension of the surface-immobilized primer-template duplexes. After acquisition of images tiling the full array, chemical cleavage and release of the fluorescent label permits the subsequent cycle of extension and imaging.

The diversity and rapid evolution of next-generation sequencing technology is post challenges for bioinformatics in areas including sequence quality scoring, alignment: assembly and data release. The topic of sequence quality scoring has become an area of intent interest, given the relatively low quality of raw data from the new sequencing platform and the various

context dependent error distributions associated with different sequencing by synthesis biochemistries. Even at this early stage of commercial availability, a variety of software tools are available for analysing next- generation sequencing data. Their functions fit into several general categories, including: (i) alignment of sequence reads to a reference; (ii) base-calling and/or polymorphism detection; (iii) *de novo* assembly, from paired or unpaired reads; and (iv) Genome browsing and annotation. Alignment and assembly represent particularly interesting problems.

Applications of new-generation sequencing include (i) full-genome resequencing or more targeted discovery of mutations or polymorphisms (ii) mapping of structural rearrangements, which may include copy number variation, balanced translocation breakpoints and chromosomal inversions; (iii) 'RNA-Seq', analogous to expressed sequence tags (EST) or serial analysis of gene expression (SAGE), where shotgun libraries derived from mRNA or small RNAs are deeply sequenced; the counts corresponding to individual species can be used for quantification over a broad dynamic range, and the sequences themselves can be used for annotation (e.g., splice junctions and transcript boundaries); (iv) large-scale analysis of DNA methylation, by deep sequencing of bisulfite-treated DNA; (v) 'ChIP-Seq', or genome-wide mapping of DNA-protein interactions, by deep sequencing of DNA fragments pulled down by chromatin immunoprecipitation. Over the next few years, the list of applications will undoubtedly grow, as will the sophistication with which existing applications are carried out.

Suggested Readings

- Hutchison, C.A., Ill. DNA sequencing: bench to bedside and beyond. *Nucleic Acids Res.* 35,6227-6237 (2007).
- Meyer, M., Stenzel, U. &Hofreiter, M. Parallel tagged sequencing on the 454 platform. *Nat. Protocols* 3, 267-278 (2008).
- Mitra, R.D., Shendure, J., Olejnik, J., Edyta Krzymanska, O. &Church, G.M. Fluorescent in situ sequencing on polymerase colonies. *Anal. Biochem.* 320, 55-65 (2003).
- Ronaghi, M., Karamohamed, S., Pettersson, B., Uhlen, M. and Nyren, P. (1996). Real-time DNA sequencing using detection of pyrophosphate release. *Anal. Biochem.* 242,84-89.
- Sanger, F., Nicklen, S. and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Science* 309, 1331-1333.
- Shendure, J. and Hanlee J. Next-generation DNA sequencing. *Nature biotech.26, 1135*-1145 (2008).
- Shendure, J., Mitra, R.D., Varma, C. &Church, G.M. Advanced sequencing technologies: methods and goals. *Nat. Rev. Genet.* 5,335-344 (2004).

Introduction to serological tests for the diagnosis of parasitic diseases – principals and applications

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Very often when presence of parasitie is very less, the conventional parasitological tests fails to detect the infection and in such cases serological tests become very helpful to detect the infection specially in chronic carrier status of the disease. Some important serological tests are:

Agglutination and Precipitation Tests: When antibodies are mixed with their corresponding antigens on the surface of large, easily sedimented particles such as animal cells, erythrocytes, or bacteria/ parasite, the antibodies cross-link the particles, forming visible clumps. This reaction is termed as agglutination. Agglutination is a serological reaction and is very similar to the precipitation reaction. Both reactions are highly specific because they depend on the specific antibody and antigen pair. The main difference between these two reactions is the size of antigens. For precipitation, antigens are soluble molecules, and for agglutination, antigens are large, easily sedimented particles. Agglutination is more sensitive than precipitation reaction because it takes a lot of more soluble antigens and antibody molecules to form a visible precipitation. To make the detection of soluble antigen and antibody reaction more sensitive, a precipitation reaction can be transformed into an agglutination reaction by attaching soluble antigens to large, inert carriers, such as erythrocytes or latex beads. Two types of agglutination tests are generally employed - i. Rapid slide/card agglutination test and ii. Plate agglutination test.

The circumoval precipitin (COP) test was used to detect serum antibodies to *Schistosoma mansoni, S. haematobium,* or both species by using eggs of either species of schistosome.

1. **Complement fixation test (CFT):** This is a classical serological test which is still considered as gold standard in many infectious disease diagnosis. In this test two principles are employed –i.) complement if fixed with antigen-antibody complex and ii). In presence of complement sheep rbc is lysed by its antibody (haemolysin). Here, antigen and antibody are allowed to act in presence of complement. Thereafter, sheep rbc and haemolysin (antibody) is added. In case of positive reaction, where antigen and antibody is corresponding each other,

complement fixed with antigen-antibody complex and later is not available so haemolysis not occurs and button of rbcs is formed. In negative case, being non-corresponding, antigen and antibody not bind and complement is available to lyse rbc and no button of rbcs is formed. Though it is very old test but till recommended by OIE for many diseases like dourine.

2. **Fluorescent Antibody Test:** In this test antigen is coated on slide and on this suspected antibody is added and further the reaction is traced with a secondary antibody conjugated with fluorescent agent. Here antibody is detected. This test needs expert observation to distinguish between specific and nonspecific signal. This test is not suitable for screening of large number of samples. Even it being an old test still recommended by OIE standard test for many parasitic diseases e.g. equine piroplasmosis, bovine theileriosis, anaplasmosis etc.
3. **Enzyme linked immunosorbent assay (ELISA):**Enzyme linked immune sorbent assay or ELISA has become very popular in diagnosis of parasitic diseases during recent past years. The first step in an ELISA experiment is the immobilization of the antigen/ antibody in polystyrene plate surface of a microtiter plate. After immobilization, a detection antibody is added, which binds to the adsorbed antigen thereby leading to the formation of an antigen-antibody complex. The detection antibody is either directly conjugated to an enzyme, such as horseradish peroxidase (HRP), or provides a binding site for a labeled secondary antibody. In general, ELISAs can be grouped into the four main categories: Direct, Indirect, Sandwich, and Competitive ELISAs.
4. **Western blot :** The mechanism applied here also like ELISA, but here reaction is done on nitro cellulose paper/ PVDF membrane and the end product is insoluble and can be fixed on the membrane.
5. **Lysis/ neutralization test:** In such test on the organism specific antibody is allowed to react and in positive cases lysis of the organism occurs. The immune trypanolysis test, a serological test detecting antibodies and revealing contact with *Trypanosoma brucei* (T.b.) gambiense, is increasingly implemented as a remote reference test to refine serological procedures of HAT surveillance under WHO programme and for managing suspect cases that are serologically positive for specific antibodies but in whom trypanosomes are not detected by microscopic observation. TL is restricted to the reference laboratories that can maintain cloned trypanosome populations expressing a particular variable antigen type. The TL test is usually

performed on plasma or serum that needs to be kept frozen until use, which impedes storage in the field and transport to the reference laboratory.

6. **Dye test:** A Sabin–Feldman dye test is a serologic test to diagnose for toxoplasmosis. The test is based on the presence of certain antibodies that prevent methylene blue dye from entering the cytoplasm of *Toxoplasma* organisms. Patient serum is treated with *Toxoplasma* tachyzoites and complements as activator, and then incubated. After incubation, methylene blue is added. If anti-Toxo antibodies are present in the serum, because these antibodies are activated by complements and lyse the parasite membrane, Toxoplasma tachyzoites are not stained (positive result); if there are no antibodies, tachyzoites with intact membrane are stained and appear blue under microscope (negative result). The dilution of the test serum at which 50% of the tachyzoites are thin, distorted and colorless is reported as antibody titer of the test serum. The test is highly sensitive and specific with no false positives reported so far.

Drawbacks of this test: 1. Difficulty in maintaining the live tachyzoites. 2. It detects immunoglobulin G(IgG) antibodies, hence cannot differentiate between recent or past infection.

Further reading:

1. Sambrook, J., Russell, D., 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York.
2. Harlow,E.D., Lane,D. 1988. Antibodies a laboratory manual. . Cold Spring Harbor Laboratory Press, New York.

Different types of ELISA with special reference to iELISA including standardization

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Enzyme linked immune sorbent assay or ELISA has become very popular in diagnosis of parasitic diseases during recent past years.The first step in an ELISA experiment is the immobilization of the antigen/ antibody in polystyrene platesurface of a microtiter plate. After immobilization, a detection antibody is added, which binds to the adsorbed antigen thereby leading to the formation of an antigen-antibody complex. The detection antibody is either directly conjugated to an enzyme, such as horseradish peroxidase (HRP), or provides a binding site for a labeled secondary antibody. In general, ELISAs can be grouped into the four main categories: Direct, Indirect, Sandwich, and Competitive ELISAs.

1. Direct ELISA:

An antigen is immobilized in the well of an ELISA plate. The antigen is then detected by an antibody directly conjugated to an enzyme such as HRP. Direct ELISA detection is much faster than other ELISA techniques as fewer steps are required. The assay is also less prone to error since fewer reagents and steps are needed, i.e. no potentially cross-reacting secondary antibody needed. Although there are some disadvantages to this method. As the antigen immobilization is not specific, higher background noise may be observed in comparison to indirect ELISA. This is primarily because all proteins in the sample, including the target protein, will bind to the plate. Direct ELISA is less flexible since a specific conjugated primary antibody is needed for each target protein. As no secondary antibody is used there is no signal amplification, which reduces assay sensitivity. Finally, the direct ELISA technique is typically used when the immune response to an antigen needs to be analyzed.

Advantages	Disadvantages
Faster than other ELISA – the technique has fewer steps	Antigen immobilization is not specific - may cause higher background noise than indirect ELISA. Mainly because all proteins in the sample, including the target protein, will bind to the plate Less flexible - each target protein needs a specific conjugated primary antibody No signal amplification - reduces assay sensitivity
Less prone to error – as less reagents and fewer steps are required	
Best for: when analyzing the immune response to an antigen.	

2. Indirect ELISA

Generally, antigen is adsorbed to a well in an ELISA plate. Detection is a two-step process. First, an unlabeled primary antibody binds to the specific antigen. Second, an enzyme conjugated secondary antibody that is directed against the host species of the primary antibody is applied.

The indirect ELISA method has high sensitivity since more than one labeled secondary antibody can bind the primary antibody; it is more economical than the direct ELISA as fewer labeled antibodies are needed. Indirect ELISA delivers greater flexibility since different primary antibodies can be used with a single labeled secondary antibody. Among its disadvantages is the possibility of cross-reactivity of secondary antibody to the adsorbed antigen, which could increase background noise. Also, indirect ELISA assays take longer to run than direct ELISAs since an additional incubation step for the secondary antibody is required. The indirect ELISA is most suitable for determining total antibody concentration in samples.

Advantages	Disadvantages
High sensitivity - more than one labeled secondary antibody can bind the primary antibody	Possibility of background noise - secondary antibody may be cross-reactive
Economical - fewer labeled antibodies are needed	Longer procedure than direct ELISA technique - additional incubation step for secondary antibody needed
Greater flexibility - different primary antibodies can be	

Advantages	Disadvantages
used with a single labeled secondary antibody	
Best for: determining total antibody concentration in samples.	

3. Sandwich ELISA

Sandwich ELISAs require the use of matched antibody pairs (capture and detection antibodies). Each antibody is therefore specific for a different and non-overlapping region or epitope of the antigen. It is important that matched antibody pairs are tested specifically in sandwich ELISA to ensure that they detect different epitopes, to achieve accurate results.
The procedure for a sandwich ELISA firstly requires the well of an ELISA plate to be coated with a capture antibody. The analyte or sample is then added, followed by a detection antibody. The detection antibody can be enzyme conjugated, in which case this is referred to as a direct sandwich ELISA. If the detection antibody used is unlabeled, a secondary enzyme-conjugated detection antibody is required. This is known as an indirect sandwich ELISA. The key advantage of a sandwich ELISA is its high sensitivity; it is 2-5 times more sensitive than direct or indirect ELISAs. Sandwich ELISA also delivers high specificity as two antibodies are used to detect the antigen. The advantages bring with them a few disadvantages; if a standardized ELISA kit or tested antibody pair is not available, antibody optimization has to be worked out since it is important to reduce cross-reactivity between the capture and detection antibodies. Sandwich ELISAs are particularly suited to the analysis of complex samples, since the antigen does not need to be purified prior to the assay yet still delivers high sensitivity and specificity.

Advantages	Disadvantages
High sensitivity - 2-5 times more sensitive than direct or indirect ELISA	Antibody optimization can be difficult - cross-reactivity may occur between the capture and detection antibodies. Needs a standardized ELISA kit or tested antibody pair.
High specificity - two antibodies are involved in capture and detection	

Advantages	Disadvantages
Flexibility - both direct and indirect detection can be used	
Best for: analysis of complex samples, since the antigen does not need to be purified prior to measurement.	

4. Competition/Inhibition ELISA

The competition/inhibition ELISA, also known as a blocking ELISA, is perhaps the most complex of all the ELISA techniques. However, each of the above assay types can be adapted to a competitive format. The competitive/inhibition ELISA is predominantly used to measure the concentration of an antigen or antibody in a sample by detecting interference in an expected signal output. Essentially, sample antigen or antibody competes with a reference for binding to a limited amount of labeled antibody or antigen, respectively. The higher the sample antigen concentration, the weaker the output signal, indicating that the signal output inversely correlates with the amount of antigen in the sample. A known antigen is used to coat a multi-well plate. Following standard blocking and washing steps, samples containing unknown antigen are added. Labeled detection antibody is then applied for detection using relevant substrates (e.g. 3,3',5,5'-Tetramethylbenzidine or TMB). If there is a high concentration of antigen in the sample, a significant reduction in signal output will be observed. In contrast, if there is very little antigen in the sample, there will be very little reduction in the expected signal output.

Advantages	Disadvantages
Main advantage - no sample processing is required and crude or impure samples can be used	Same limitations as base ELISA - as each ELISA technique can be adapted to a competitive format
More robust - less sensitive to sample dilution and sample matrix effects than the sandwich ELISA	
More consistent - less variability between duplicate samples and assays	

Advantages	Disadvantages
Maximum flexibility - it can be based on direct, indirect or sandwich ELISA	
Best for: commonly used when only one antibody is available for the antigen of interest. It is also suitable for detecting small antigens that cannot be bound by two different antibodies such as in the sandwich ELISA technique.	

Besides, different types of ELISA can be exploited to detect antigen/antibody;so the researcher can modify the test as per their necessity.

Further reading:

- Sambrook, J., Russell, D., 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York.
- Harlow,E.D., Lane,D. 1988. Antibodies a laboratory manual. . Cold Spring Harbor Laboratory Press, New York.

Usefulness of Monoclonal Antibodies in Diagnosis and Therapeutics

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Introduction: Kohler and Milstein in 1975 revolutionized serology on publishing their research article on the generation of monoclonal antibodiesusing Hybridoma Technology. They described the protocol for immortalizing single antibody producing B cells by fusing them with myeloma cells, enabling one to obtain limitless amounts of an antibody from a single clone of B cells, over indefinite periods of time. Since then, several technologies have been reported for making monoclonal antibodies (mAbs) such as, phage display libraries, single B cell culture, single cell amplification from B cell populations, etc. Molecular biology approaches are now being used to amplify the heavy and light chains of the antibody genes and employing the eukaryotic or the prokaryotic protein expression systems to produce antibodies or single chain fragment variables (scFv).

Over the last several years, we have generated a large number of mouse mAbs in our laboratory, using the conventional hybridoma technology. The antibodies have various utilities, like, purification and functional characterization of proteins of interest, cell entry inhibition of viruses, neutralization of cytotoxic agents and conjugates for cell-targeted therapy. This article describes a few antibodies that maybe explored for use in diagnostics and therapeutics.

Monoclonal antibodies to Glycodelin:

The fetus expresses paternal allo-antigens and is regarded as non-self by the maternal immune system, but for a handful of indispensable factors that protect it from rejection to sustain pregnancy. One such molecule Glycodelin-A (GdA), secreted by the endometrial glands under progesterone regulation was shown to inhibit the proliferation of T cells. We proposed to delineate the immunosuppressive activity of GdA at the molecular level for which obtaining purified native GdA from the amniotic fluid was necessary and became a challenge because of the abundance of serum albumin and globulins in the fluid. The Gd gene was cloned and expressed in *P pastoris*, purified, mAbs generated and characterised. A mAb with intermediary affinity for binding to GdA was chosen, ascites was generated and the purified antibody was conjugated to hydrazide Sepharose beads for use in affinity chromatography which served as a

one-step purification method for obtaining a homogeneous preparation of GdA from amniotic fluid. Using high affinity mAbs to GdA, we established a radio-immunoassay for the estimation of Gd in different body fluids. We are currently in the process of establishing an ELISA for the same.

Monoclonal antibodies to abrin:

The plant toxin Abrin, a type II ribosome inactivating protein (RIP), is highly lethal (LD_{50} ~ 1 µg/Kg body weight) and is listed under 'bioterrorist weapons'. Towards establishing abrin-neutralizing reagents we obtained two high affinity mAbs that protect cells from abrin-induced toxicity and more importantly protect mice against lethal doses of the toxin. The epitope corresponding to the two mAbs were mapped to understand the mechanism of neutralization.

Monoclonal antibodies to GnRH receptors as cell-targeted therapy

Immunotoxins, constructed by conjugating cell-surface receptor-specific antibodies to a lethal toxin, are being considered for treatment of cancer in combination therapy. The RIP, Abrin, from the plant *Abrus precatorius*has a very high catalytic efficiency. Apart from inhibiting protein synthesis, Abrin also induces apoptosis, which makes it a good candidate for cancer therapy. We conjugated the recombinant toxin A chain of abrin to an antibody raised against human Gonadotropin releasing hormone receptor (GnRHR) which are over expressed in a few gynecological cancers. The immune conjugate inhibited protein synthesis and also induced apoptosis specifically in cells expressing GnRHRs. Our studies provide the proof of concept for utilization of Abrin in cell-targeted killing.

Monoclonal antibodies to the envelope protein of hepatitis C virus

Hepatitis C virus (HCV) represents a major global health threat. The envelope glycoproteins, E1-E2 of HCV play an important role in infection by binding to hepatocyte surface receptors leading to virus entry. Identification of antigenic determinants in these domains would aid in the development of anti-virals. We aimed to delineate neutralizing epitopes of HCV envelope proteins. Using HCV-like particles (HCV-LPs) corresponding to genotype 3a (prevalent in India), we obtained three mAbs specific for the E2 protein that significantly inhibited virus binding to hepatoma cells. The epitope of one mAb overlaps with the CD81 receptor-binding site

and that of another, with the hypervariable region 2 of the E2 protein. A combination of these antibodies significantly inhibited HCV binding and entry in both HCV pseudo particle (*in vitro*) and HCV cell culture (*ex vivo*) system compared to the mAbs alone.

Monoclonal antibodies to human Tumor necrosis alpha

Tumor necrosis alpha (TNFα) is a pro-inflammatory cytokine having pleiotropic effects on immune cells and plays an indispensable role in inflammation, cell differentiation, cell proliferation, apoptosis and cell metabolism.Although TNFα plays an essential role in an effective immune response, the unrestricted production of it may lead to inflammatory disorders, like, diabetes, cancer, rheumatoid arthritis etc. Inhibition of TNFα by pharmacological agents has proven to be effective in palliative treatment.

We developed an anti-human TNFα mAb which can neutralize TNFα activity and is highly comparable to Infliximab (a commercialized anti-TNFα antibody). We constructed scFv from the hybridoma cells with the idea to use the smaller molecule in the management of inflammatory disorders.

APPLICATIONS OF NANOPARTICLES IN DIAGNOSTICS

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Introduction: The increasing presence of contaminants in food, air or drinking water that are capable of causing intoxication, diseases or chronic illness, has stressed the need for analytical systems capable of rapid multi-analyte measurements of complex samples. Similarly, multi-parameter diagnostic systems are increasingly required in order to detect all the well-known and the more recently appeared biomarkers for different diseases. When the detection system requires a bio-molecular recognition event, antibody-based detection methodologies are still considered the standard assays in environmental, food and clinical analysis. These assays are well established and they have been demonstrated to reach the desired sensitivity and selectivity. However, the use of antibodies in multi-analyte detection methods and in the analysis of very complex samples could encounter some limitations mainly deriving from the nature and synthesis of these protein receptors. In order to circumvent some of these drawbacks, other recognition molecules are being explored as alternatives.

Pathogen Recognition Elements

The recognition elements used in whole cell biosensors are generally biomolecules that have an affinity for epitopes present on the pathogen surface. They include proteinaceous antibodies, nucleic acid aptamers, carbohydrates antimicrobial peptides and molecularly imprinted polymers.

Antibodies. Antibody-based methods have been used extensively to detect bacteria, virus, toxins, and spores, alike. Highly selective and sensitive antibodies are readily available for many pathogens, and there are well-established methods to conjugate antibodies to nanomaterials. For these reasons, immunological recognition by antibodies continues to be the most widely used tool for the selective capture and labeling of microorganisms. Three categories of antibodies are used in immunoassays: polyclonal, monoclonal and engineered antibody fragments. Polyclonal antibodies (pAbs) are produced in vivo and consist of a suite of antibodies that bind to a number of epitopes on the antigen. Monoclonal antibody (mAbs) solutions are produced in vitro from hybridoma cell lines and consist of an identical, well-defined population of antibodies that bind to a single epitope.

Carbohydrates. Carbohydrates (oligosaccharides or polysaccharides) are a large and diverse class of biomolecules that play an important role in dictating pathogen (and toxin) recognition and attachment to human cells. Many pathogens as well as their toxins recognize and bind to specific carbohydrate sequences in the glycocalyx. For example, type I fimbriae on Enterobacteriaceae bind to mannose terminated glycoproteins, influenza virus binds to N-acetyl neuraminic acid and rotavirus binds to galactose.

Antimicrobial Peptides. Antimicrobial peptides (AMPs) play an integral role in the immune system response to pathogen infection. Consisting of sequences of 15-45 amino acids. AMPs recognize and semi selectively bind to microbial surfaces and, for reasons that are not completely understood, facilitate pathogen lysis. Similar to carbohydrates, AMPs exhibit a range of activity toward bacteria, virus and fungi and have been suggested for use in sensor arrays that incorporate multiple AMPs. AMPs targeting Gram-negative bacteria bind nonspecifically to the negatively charged lipopolysaccharide of both pathogenic and nonpathogenic organisms, while AMPs targeting Gram-positive bacteria often target peptidoglycan precursors required for synthesis of the bacterial cell wall.

Aptamers.

Detection of biological terrorist threat agents, such as bacterial cells, spores, viruses and toxins, is a significant military and civilian challenge. Traditional analytical techniques for these targets are mainly based on immunological methods (Peruski and Peruski, 2003) such as conventional ELISA, immunomagnetic-electrochemiluminescence assays (Gatto-Menking et al., 1995) or time-resolved fluorescence assays (Peruski et al., 2002). However, in this particular field, aptamers can be of great advantage since all these methods are very dependent on the possibility of producing specific antisera for these toxic materials in animals. Aptamers due to their synthetic nature are independent of animals and they can be selected also for these toxic molecules. Aptamers specific for targets, such as anthrax spores, cholera toxin, staphylococcal enterotoxin B, ricin and abrin toxin, have been selected (Bruno and Kiel, 1999, 2002; Kirby et al., 2004; Tang et al., 2007) and by using these aptamers different detection systems have been developed.

Aptamers are single stranded DNA or RNA ligands which can be selected for different targets starting from a huge library of molecules containing randomly created sequences (Tombelli et al., 2005); or peptide molecules that bind to a specific target molecule, but natural aptamers also

exist in riboswitches. Aptamers can be used for both basic research and clinical purposes as macromolecular drugs. The selection process of Aptamers is called systematic evolution of ligands by exponential enrichment (SELEX), first reported in 1990 (Ellington and Szostak, 1990; Tuerk and Gold, 1990). The SELEX process involves iterative cycles of selection and amplification starting from a large library of oligonucleotides with different sequences (generally 1015 different structures). After the incubation with the specific target and the separation of the binding from the non-binding molecules, the oligonucleotides that are selected are amplified to create a new mixture enriched in those nucleic acid molecules having a higher affinity for the target. After several cycles of the selection process, the pool is enriched in the high affinity sequences at the expense of the low affinity binders.

Recognition properties of Aptamers:

Conjugation of aptamers to either lipids or polymers such as polyethylene glycol improves their stability and distribution kinetics sufficient to produce therapeutic effects. The molecular recognition properties of aptamers are very similar to antibodies, which recognize a target with high affinity and specificity and in many cases effectively inhibit its function. Some of the best aptamers form complexes that have dissociation constants in the picomolar range, while many have dissociation constants that are similar to the antigen-binding fragment of antibodies. In terms of selectivity, aptamers can discriminate between very subtle structural differences, such as the presence or absence of a hydroxyl group or structural enantiomers (mirror images that have an identical chemical composition) of the target. Due to their relatively small size compared with antibodies, aptamers can fit into clefts where bulky molecules such as antibodies would otherwise be excluded. Their flexibility allows them to fold and assume the shape of relatively small binding pockets, thereby maximizing surface contact with the target protein. These desirable properties of aptamers, combining the optimal characteristics of small molecules and antibodies, show great promise and have opened avenues for the development of therapeutic, antiviral, diagnostic and targeted drug delivery tools in areas that have been hitherto refractory to other approaches

Advantages of Aptamers.

1. The main advantage is the overcoming of the use of animals or cell lines for the production of the molecules.

2. Antibodies against molecules that are not immunogenic are difficult to generate, whereas, toxins and molecules that do not elicit a good immune response and are not suitable targets for immunotherapy can be used as targets for the generation of high-affinity aptamers. Furthermore, aptamers are isolated by in vitro methods that are independent of animals.

3. In addition, generation of antibodies in vivo means; the animal immune system selects the sites on the target protein to which the antibodies bind. The in vivo parameters restrict the identification of antibodies that can recognize targets only under physiological conditions limiting the extension to which the antibodies can be functionalized and applied.

4. Moreover, the aptamer selection process can be manipulated to obtain aptamers that bind a specific region of the target and with specific binding properties in different binding conditions.

5. After selection, aptamers are produced by chemical synthesis and purified to a very high degree by eliminating the batch-to-batch variation found when using antibodies. By chemical synthesis, modifications in the aptamer can be introduced enhancing the stability, affinity and specificity of the molecules. Often the kinetic parameters of aptamer–target complex can be changed for higher affinity or specificity.

6. Another advantage over antibodies can be seen in the higher temperature stability of aptamers; in fact antibodies are large proteins sensitive to the temperature and they can undergo irreversible denaturation. On the contrary, aptamers are very stable and they can recover their native active conformation after denaturation.

7. Aptamers offer advantages over antibodies and other conventional molecules as they can be entirely produced in a test tube using enzymes, or can be readily produced by chemical synthesis within days rather than by tedious biological expression used to make antibodies. The in vitro process or chemical synthesis used to make aptamers represents a rapid, low cost and less batch-to-batch variation process than the in vivo process used for production of antibodies.

Limitations of Aptamers

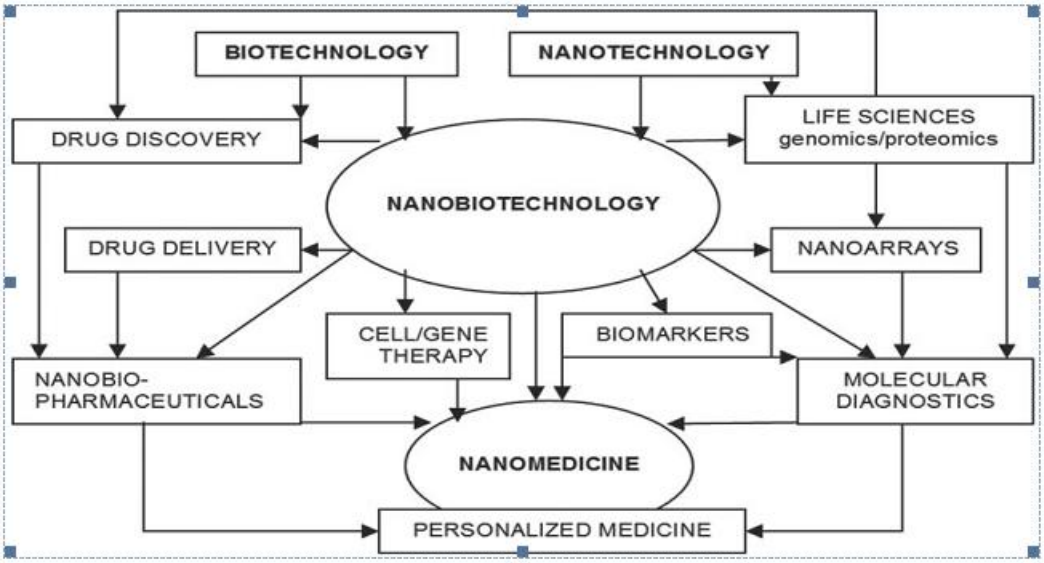
The primary limitation on the use of aptamers (mainly RNA aptamers) in bioanalytical methods has been their nuclease sensitivity which is very critical for their use in ex vivo and in vivo applications (Famulok et al., 2000). However, it has been shown that the stability of such molecules can be improved by chemical modification of the ribose ring at the 20-position (Pieken et al., 1991). A different approach to stabilize aptamers comes from selection of RNA aptamers binding to stereoisomers of an intended target molecule, followed by chemical

synthesis of the mirror-image of the selected sequences. As a consequence of molecular symmetry, the mirror-image aptamer (L-ribose) binds to the natural target molecule. Because of the substitution of the natural D-ribose with L-ribose, the mirrorimage aptamer is totally stable (Klussmann et al., 1996).

Nanoscience and Nano particles

Nanotechnology or nanoscience is a highly multidisciplinary field of applied science and technology intended to create, understand and use atomic and molecular scale (0.1-100nm) structures and to fabricate devices or materials that lie with in the nano size range.

It applies the principles of engineering, electronics, physical and material science, and manufacturing to the molecular or submicron level.



This scheme shows the interrelationship of various technologies that contribute to clinical nano diagnostics. These technologies also contribute to development of nanomedicine under the concept of personalized medicine.

Nanoparticles (NP) are defined as particulate dispersions or solid particles with a size in the range of 10-1000 nm. The application of Nanotechnology to disease treatment, diagnosis, monitoring and to the control of biological systems has recently been referred to as nanomedicine. The medical uses of nanomaterials include advanced drug delivery systems, new therapies and invivo imaging. It offers a suitable means of delivering small molecular weight NP

drugs, as well as macromolecules, such as proteins, peptides or genes by either localized or targeted delivery to the tissue of interest.

Nano materials used for drug delivery must meet several requirements such as- biocompatibility, drug compatibility, suitable biodegradation kinetics, mechanical properties and ease of processing.

Multifunctional nanoparticles:

The nanoparticle's "corona" can be functionalized with hydrophilic polymers, targeting molecules, therapeutic drugs, and image contrast agents. The interior core can be solid (e.g., quantum dots) or liquid (e.g., liposomes).

Nanomaterials: Three different groups of nanotechnology materials are raw materials, nanostructured materials and materials such as nanotubes and fullerenes. The raw materials include nanoparticles and nanocrystalline materials which are more effective than bulk materials. Commonly used nanomaterials are fullerenes, nanotubes, buckeyballs, quantum dots, dendrimers, nanoshells etc., Nanomaterials compared to the macro materials can have very different properties. They can be stronger, lighter, more electrically conductive, more porous and less corrosive than bulk materials. They can even change colour viz., gold can appear red, blue or gold depending on their size. Inorganic nonmaterial can detect electrical changes in biological molecules and help in detecting or treating a disease.

Buckeyballs: They are pure carbon molecules composed of 60-80 atoms of carbon. Because a fullerene takes a shape similar to a soccer ball or a geodesic dome, it is sometimes referred as Fullerene after the inventor of geodesic dome, Buckminister Fuller. In the buckeyball each carbon atom is bonded to three of its neighbors. They are mainly used in delivery of medicine or radioactive material to a disease site.

Nanotubes: Nanotubes are essentially buckey balls that have been opened on two sides with additional atom groups added in the characteristic hexagon shape to form a hollow carbon tube (cylinder). They are related to other form of carbons such as graphite and diamonds. They are sheets of graphite rolled into a cylinder. Nanotubes are also called buckeytubes.

Quantum dots

Among various nanomaterials, quantum dots (QDs) distinguish themselves in their far-reaching possibilities in many avenues of biomedicine. QDs are nanoscale fluorescent semiconductor crystals with unique photochemical and photo physical properties. Their much greater

brightness, rock-solid photo stability and unique capabilities for multiplexing, combined with their intrinsic symmetric and narrow emission bands, have made them far better substitutes for organic dyes in existing diagnostic assays.

In biological applications QDs have advantages over the traditional organic fluorophores due to their narrow, symmetric emission spectra, while requiring only a single excitation wavelength to simultaneously resolve multiple photostable colors. The unique properties of QDs have enabled multiplexed imaging of cellular targets for studying cancer biology, multiphoton fluorescence studies for deep tissue imaging in live animals and near-infrared imaging for sentinel lymph node mapping at 1 cm tissue depth.

Dendrimers (Polymers): These are synthetic three dimensional man made macromolecules formed using a nanoscale fabrication. The unique features of the dendritic architecture include a high degree of structural symmetry, intramolecular minimum value and a well defined number of terminal groups.

Nanoshells: Nanoshells are colloids that consist of a core of non-conducting material covered by a thin metallic shell. By varying the thickness of metal shell, researchers can precisely tune the color of light to which the nanoshells respond. The infra red is suitable for whole blood immunoassay as it easily penetrates the whole blood well. When the antibody nanoshell particles are placed into a solution of whole blood containing the test molecules, it causes slight changes in the optimal properties of the nanoshells. By monitoring the changes, it is possible to detect the slight concentration of antigens in the blood.

Cantilevers: Cantilevers are made of silicon nitrite coated with gold on one surface, are mechanical beams anchored at one end and free standing at the other, similar to a swimming pool diving board. The cantilever bends in response to the change in surface stress upon binding of target molecule from a body fluid such as serum. The bending can be measured both optically and electrically which can be scaled up to an array format with as many as hundreds of cells for simultaneous detection of multiple biomarkers requiring minimal clinical samples.

Applications:

Disease diagnosis: Nanotechnology can be used in making cheaper, faster and more precise diagnostic tools. The nanotechnology can improve the quality of images produced by ultrasound machine. Nanoparticles injected into the breast can help the doctors to detect the cancers at very

early stage. Nanotechnology based on the gold nanoparticles and DNA can detect prostrate specific antigen in the blood when present at extremely low levels.

In the conventional immunoassay, whole blood cannot be used as it is so viscous and murky that it interferes with the chemical reaction in the test. By adopting nanotechnology, researchers have made it possible to test whole blood by using optically active gold-coated glass particles commonly known as gold nanoshells. The nanoshell immunoassay can detect less than one billionth of a gram of IgG in 1 ml of whole blood in 30 minutes.

Future blood tests may use tiny bar codes to speed up disease diagnosis like scanning a bar code of a grocery item. Unique DNA tags called bio-bar-codes can be used to detect the disease markers. The tags can be scanned by an instrument to identify disease starting from Alzheimer's disease to bio-terror agents such as anthrax, Ebola, Marburg or small pox. The test is easier, faster, more accurate and less expensive than PCR. The new test called bio-bar code amplification could be ready shortly and a drop of blood is enough to screen the patient against a number of diseases.

Treatment: The nanoshells with targeted agent are injected into all animals and after a week animal's body is illuminated with infrared to raise the cell temperature to about 55oC to activate the cancer killing agents to destroy the tumor. The smart superparamagnetic nanoparticles made up of iron oxides injected into the blood stream target tumor receptor cells when subjected to a magnetic field by emitting an attached drug. Quantum dots may also be injected into the blood stream of animals and upon stimulation with light capable enough to kill the cancerous cell. Nucleic acid engineered probes and methods offer powerful new ways to deliver therapeutics on preventive treatment for particular disease. The major challenge is to develop a non-viral DNA delivery system that has low toxicity and cost but high level of efficiency and specificity.

Identity preservation: Identity preservation (IP) system is a system that provides consumers with information about the practices and activities used to produce an agricultural product. Quality assurance of the safety and security of agricultural products could be significantly improved through IP at the nanoscale level. Nanoscale IP has the potential to continuously track and record the history of a particular agricultural product. The keys are biodegradable sensors for temperature and other stored data to track all stages in the life of the product including the birth of the animal, its medical history, the slaughter house, meat packing plant, right through to the consumer's table.

Animal breeding: The management of breeding is an expensive and time consuming problem in dairy and swine industry. The nanotube implanted under the skin will provide the information about the level of estradiol in the blood during oestrous in animals by near infrared fluorescence. The signal from this sensor will be incorporated in a central monitoring and control system to inseminate the animals for improving the conception rate/breeding performance.

Drug delivery: Nanomaterials such as buckeyballs and dendrimers can be used in drug delivery systems. Buckey balls are inert, non-toxic perfectly smooth and can interact easily with cells, protein and viruses. Additionally they are hollow inside where drugs can be put so that it can release the drugs inside the cells. Dendrimers are synthetic polymers in various predetermined sized and can be used as delivery vehicle as it can hold a drug inside. They can enter cells .very easily and release drugs right on target. They do not trigger immune response and execute a five step task while dealing with the treatment of tumours (1) dendrimers may able to find tumour cells in the body by looking for tumour receptors (2) bind and pass through cell membrane (3) perform chemical analysis to know the type of tumour (4) release chemotherapy or radioactive agents inside the cells (5) confirmation of the death of tumor cells by chemical analysis.

Besides, targeting tumor cells the drug delivery systems, dendrimers showed promising results as tools in MRI and gene transfer techniques. Dendrimer based nanocomposites are being studied as possible anti-microbial agents against *Staphylococcus aureus*, *Pseudomonas aeroginosa* and*E. coli*. Nanotechnology has also entered the field of vaccinology. Synthetic oligonucleotides and antigens in bio-degradable nanospheres can be used as an alternative approach for immunization. A better immune response seems to be obtained with biodegradable nanospheres than with vaccines produced by conventional methods.

Now-a-days antibiotics, probiotics and pharmaceuticals are delivered to animals primarily through feed or injection. The medicine is delivered as a preventive measure or as a treatment once the disease organism has multiplied and symptoms are evident. Nanoscale devices have the capacity to detect and treat an infection, nutrient deficiency or other health problem long before symptoms are evident at the macroscale. This type of treatment could be targeted to the affected area and have multifunctional characteristics viz., time controlled, spatially targeted, self-regulated, remotely regulated and pre--programmed. Smart delivery systems can also have the capacity to monitor the effects of the delivery of pharmaceuticals, neutraceuticals, nutrients, food supplements, bio-active compounds, probiotics, chemicals and vaccines.

Toxicity: The application of nanotechnology in different fields is not free from drawbacks. The particle size and surface are important characteristics when considering the toxicity of a material. As the size of the particle decreases, the surface area increases exponentially which allows for more potentially reactive groups to interact with the environment on the surface. It has been well established fine particles in the air can increase morbidity and mortality from pulmonary and cardiovascular diseases with long and short term effects. For example, exposure of human keratinocytes to carbon nanotubes was associated with oxidative stress and apoptosis. However, not all nanotubes are composed of same functional groups and nanomaterials with appropriate coating will have minimal toxicity. On the other hand, when injecting nanomaterials into humans as contrast agents, therapeutic carriers or sensors, one has to consider the rate of clearance. Iron oxide nanoparticles, for example, have been used as contrast agents and can be ingested by living cells and the biodegradation of the particles results in free iron that can be incorporated into Hb and the body is free of residues of iron oxide nanoparticles after months. Quantum dots encapsulated with the best protective shells will slowly break down in the body and eventually expose the core and release toxic ions.

References:

- Bruno, J.G., Kiel, J.L., 1999. In vitro selection of DNA aptamers to anthrax spores with electrochemi luminescence detection. Biosens.Bioelectron. 14, 457–464.
- Ellington, A.D., Szostak, J.W., 1990. In vitro selection of RNA molecules that bind specific ligands. Nature 346, 818–822.
- Famulok, M., Mayer, G., Blind, M., 2000. Nucleic acid aptamers—from selection in vitro to application in vivo. Acc. Chem. Res. 33, 591–599.
- Gatto-Menking, D.L., Yu, H., Bruno, J.G., Goode, M.T., Miller, M., Zulich, A.W., 1995.Sensitive detection of biotoxoids and bacterial spores using an immunomagnetic electrochemi-luminescence sensor.Biosens.Bioelectron. 10, 501–507.
- Kirby, R., Cho, E.J., Gehrke, B., Bayer, T., Park, Y.S., Neikirk, D.P., McDevitt, J.T., Ellington, A.D., 2004.Aptamer-based sensor arrays for the detection and quantitation of proteins.Anal.Chem. 76, 4066–4075.
- Klussmann, S., Nolte, A., Bald, R., Erdmann, V.A., Furst, J.P., 1996. Mirrorimage RNA that binds D-adenosine. Nat. Biotechnol. 14, 1112–1115.

- Kotia, R.B., Li, L., McGown, L.B., 2000. Separation of nontarget compounds by DNA aptamers.Anal. Chem. 72, 827–831.
- Peruski, A.H., Johnson, L.H., Peruski Jr., L.F., 2002. Rapid and sensitive detection of biological warfare agents using time-resolved fluorescence assays. J. Immunol. Methods 263, 35–41.
- Peruski, A.H., Peruski Jr., L.F., 2003. Immunological methods for detection and identification of infectious disease and biological warfare agents.Clin.Diagn. Lab. Immunol. 10, 506–513.
- Pieken, W., Olsen, D.B., Benseler, F., Aurup, H., Eckstein, F., 1991.Kinetic characterization of ribonuclease-resistant 20-modified hammerhead ribozymes. Science 253, 314–317.
- Tang, J., Yu, T., Guo, L., Xie, J., Shao, N., He, Z., 2007.In vitro selection of DNA aptamer against abrin toxin and aptamer-based abrin direct detection.Biosens.Bioelectron. 22, 2456–2463.
- Tombelli, S., Minunni, M., Mascini, M., 2005. Analytical application of aptamers. Biosens.Bioelectron. 20, 2424–2434.
- Tuerk, C., Gold, L., 1990. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science 249, 505–510.

Weather related livestock disease forecast and their preventive measures in different agroclimatic zones of India

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Introduction: Environmental change, demography, movements of animals and insufficient veterinary services have all contributed to the changing spectrum of infectious diseases with which global community is challenged. Existing mechanisms for infectious disease surveillance and response are inadequate to meet the increasing needs for prevention, detection, reporting and response. The ability to predict epidemics will provide a mechanism for governments and veterinary health care services to respond to outbreaks in a timely fashion, enabling the impact to be minimized and limited resources to be saved (LaPorte, 1993; Wilson, 1994). Advanced surveillance and modelling technologies incorporating environmental data provides potential to predict the temporal and spatial risk of epidemics for many infectious diseases in India. These technologies combined with communication techniques provides an important tools that are both cost-effective and timely. As disease boundaries shift and expand to threaten new populations, there is increasing need to develop operational models with predictive potential, geographical information systems are likely to play an increasingly important role in forecasting outbreaks, especially those of vector-borne diseases (Greenwood, 1998).

Advancement of livestock disease surveillance systems, sampling strategy, epidemiological modelling, and information technology generated the expectation that an early warning systems are not only feasible but necessary tools to combat the incidence of infectious diseases. The length and spatial extent of epidemiological data series are important for investigating annual and seasonal pattern of disease incidence, particularly when the availability of environmental data either free or low cost and the quality and availability of epidemiological data vary enormously. The availability of measurement technologies for satellite data and their use in the disease prediction plays in important role improving the accuracy of models.

Increasingly, there is a perceived need to exploit information from multiple sources in epidemic modelling, ensuring decision making on public health policies aimed to control epidemics is progressively based on as many diverse source of information as possible and the use of models (Rutherford et al., 2010). Policy makers want defendable models that not only

realistically approximate the phenomenon of interest, but are also, crucially, able to produce outputs consistent with all available relevant data (Rolka et al., 2007; Lipsitch et al., 2011). This requirement, supported by the continued progress in computational power, has encouraged the development of increasingly complex models, which in turn, require rich arrays of data to guarantee parameter identifiability (Ferguson et al., 2006).

Geographic Information System

Place is an important element in understanding the geographic distribution of livestock diseases and means of formulating and testing etiological hypothesis. The term ‘place ‘is usually considered as surrogate for interaction of genetic factors, species type or host and environment. In the prospective of veterinary health practices, knowledge that the veterinary health issues is concentrated in identifiable places is essential for the efficient distribution of resources for prevention, minimizing the disease burden or prevalence, treatment or amelioration. In recent years, availability of geographical information system and databases provide the excellent environment to explore the disease pattern in the given study area.

Age, breed, species type, environment material and other variables vary from one place to another and hence influences the varying risk of disease incidence. The observed differences of risk of diseases is likely to be confounded by these variables and hence the comparison of risk variability is an important issue in the process of disease epidemiology. The adjustment of potential confounding variables is important for evaluation of spread of risk of disease incidence.

National Surveillance and disease data collection:

Surveillance in the country is needed to understand the health status of the animals in the country, so that problems can be identified and action can be initiated. However, different countries have different surveillance needs and capabilities, a wealthy country with few diseases that depends on exports of animal and animal products will have specialized surveillance systems to protect trade. A poor country with uncontrolled land borders with other countries that have regular outbreaks of epidemic diseases will maintainthe specialized surveillance system and will aim at minimizing the impact of major animal diseases. There are four major reasons why surveillance activities are undertaken.

1. Demonstrating freedom from diseases, 2. Early detection of diseases, 3.Measuring the level of diseases and 4. Finding cases of disease

Sampling Plan

In a large population where animals are separated into herds (villages can be treated as herd in some country where herd information is not available), disease has a strong tendency to cluster. This is because the disease agent or agents (whether infectious, environmental or genetic) are generally not evenly distributed throughout the population.. With rare diseases, this clustering is usually even more pronounced. As a result, a very low proportion of herds may be affected by a particular disease-but within those affected herds, the prevalence of the disease amongst animals may be quite high. If a survey designed to detect the presence of disease fails to take into account the clustering of disease in the population, the results of the survey are likely to be very unreliable. This is because the probability formulae that the surveys are based on assume that every unit in the population has the same probability of being affected. Another problem with large-area surveys is the logistics of sampling. Probability formulae assume simple random sampling. Simple random sampling of individual animals from a national herd requires the creation of a sampling frame which may need to list millions of animals (each uniquely identified). Such a sampling frame is usually impossible to construct.

The solution to both these problems is to use a two-stage sampling strategy in which herds form the first stage, and individual animals within selected herds, the second stage. In this way, the sample sizes at each stage can be adjusted to reflect the different disease prevalence's (the proportion of herds affected in the first stage, and the proportion of animals affected in the herd at the second stage). Two-stage sampling also means that the construction of sampling frames is much simpler. At the first stage, only a list of all herds in the population is required, and at the second stage, only animals in each of the selected herds is to be included in the list. However, the use of two-stage sampling presents particular problems for sample-size calculation and analysis.

The use of two-stage sampling has evolved to meet surveillance objectives for two reasons. First, list of frames of animals for randomized sample selection do not typically exist at a regional or national level, but the list frames of herds can be developed and maintained more readily. Secondly, the theory and applications of within-herd sampling with imperfect diagnostic tests is well developed. The within-herd sampling research has guided the approach to sampling to classify the herd`s disease or infection status.

Data for Spatial analysis

Point and layer (area) data are two important types of spatial data. Each item in the veterinary health data system viz., population, breed, environmental exposure, morbidity and mortality

relate to a point data while data like village, herd, state, districts, blocks etc. indicated as layer data. The data for spatial analysis comes from different sources, it is advisable to collect these data taking help of veterinary epidemiologists or Bio-statisticians. It is necessary to ensure that precise and complete point or area data are used in spatial epidemiology. The data related to diagnosis, collection, coding and reporting of a given health events may vary between geographical regions and time.

The quality of data must be given important in spatial analysis of data, presence of missing cases, underreporting, under ascertainment of cases, inaccurate baseline population data, may lead to inaccurate or misleading interpretation of estimated high or low risk. The confidentiality is also an important issue in spatial epidemiology, breaching the confidentiality may cause concern, particularly when it discloses the area with high rates of morbidity/mortality, outbreaks or risk of disease incidence.

Disease mapping

Mapping of disease events is one of the best methods for better visualization for exploring the complex structure of data. Data visualization is not only creating interest and also attract the attention of viewer and provide the way for discovering the pattern. Disease mapping is one of the tools of geographical epidemiology, fulfilling the need to generate accurate and precise maps of disease events. For Example, dot or dot density maps are used display point data, while areal data were presented by Choropleth maps and for continuous surface data, contour maps or isopleth maps are used. In the veterinary epidemiology, the presentation of maps is established as a basic tool for analysis and interpretation. The selection of appropriate of administrative unit for mapping viz., village, block, district or state, selection of suitable data classification in the map viz., high risk, moderate risk, low risk no risk etc., and selection of colour schemes are the important issues associated with creation of disease maps.

Ecological analysis

Ecological analysis refers to the analysis of association of disease incidence or outbreak with covariates of interest viz. anthropogenic variables, socio-economic variables, host related information, environmental variables and remote sensing variables etc. In the ecological analysis, the variables are defined on aggregated groups of individuals rather than individuals themselves. In the ecological analysis, the important issue to be attended is scale of covariate. The optimum choice of scale is a trade-off between making the groups (regions) large enough to have stable

estimates and small enough to make them homogenous in terms of their socio-economic and other important covariates. For large regions, there is greater possibility that associations measured at the aggregate level will differ from the same association measured at individual level. This can lead to a problem known as ecological fallacy, or ecological bias, in this scenario, inference is made incorrectly at individual -level association from one that is observed at regional level. On other hand, if the regions or units chosen are too small, the results may show spurious spatial correlation or pattern due to random variation in small number of disease events.

The uncertainty induced by the aggregation procedure may result from scale dependency data and cause modifiable unit problem. Hence it is important to consider the scale of analysis of ecological variables and analyze the data at two or more levels of aggregation. It is possible to overcome the issues like scale dependency and ecological bias, by the se of multi-level approach using individual level and group level together.

Disease modelling

A disaster, precipitated by a natural hazard, can be defined as a "serious disruption of the functioning of community or society or livestock system causing widespread human, animal, material, economic or environmental losses which exceed the ability of the affected community or society to cope using its own resources. The expression of 'early warning' is used in many fields to mean the provision of information on an emerging dangerous circumstance where the information can enable action in advance to reduce the risks involved. Early warning systems exist for natural geophysical and biological hazards, complex socio-political emergencies, industrial hazards, and many other related fields.

The ability to detect outbreaks or incidence of infectious diseases early is important to minimize morbidity and mortality through timely implementation of disease prevention and control measures. The attack on world trade center and Anthrax terrorist attacks in 2001 as well as recent West Nile virus , SARS outbreaks, Nipah virus outbreaks, have motivated many public health authorities to develop early disease outbreak detection systems using non-diagnostics information, often derived from the historical data collected from other sources including environmental data. Emerging infectious diseases poses a growing threat to livestock population, climate changes like increased temperature and altered rainfall distribution are likely to increase the infectious disease particularly vector-borne diseases resulting in to emergence of zoonotic

diseases. Many of the world`s epidemics are known to be highly sensitive to changes in climate and short term fluctuations in the weather (Mills JN et.al, 2010; Gale RT et.al, 2009; Thornton PK, et.al, 2009).

Forecasting is the monitoring of specific risk parameters helping to predict situations that could lead to the occurrence of a given disease and its subsequent spread and forecasting of diseases helps to predict the course of disease, warn livestock authorities for preparedness.

Early identification of an infectious disease outbreak is an important first step towards implementing effective disease interventions and reducing resulting mortality and morbidity. The geographic and seasonal distribution of many infectious diseases are associated with climate and therefore the possibility of using seasonal climate forecasts as predictive indicators in disease early warning system (EWS) is an interest of focus. Geographic Information system(GIS), remote sensing (RS) and Global Positioning system(GPS) are the three commonly used veterinary geo-informatics technologies employed in this digital era for rapid communication of data for better management of animal diseases.

Number of models were fitted to data and tested for accuracy in terms of discrimination power. Two regression models viz. Generalized linear models(GLM) and Generalized additive models (GAM) and six Machine learning methods viz. Random Forest (RF), Boosted Regression Tree (BRT), Artificial Neural Network (ANN), Multiple Adaptive Regression Spline(MARS), Flexible Discriminant Analysis (FDA) and Classification Tree Analysis (CTA) were generally employed. In disease modelling. Different modelling methods return different type of `model object', all these model objects could be used to with the predict function to make predictions for any combinations of values of independent variables.

Spatial autocorrelation

Spatial autocorrelation refers to lack of independence of neighboring areas. The correlation or dependency refers to the index for geographically close areas are more related than those areas that are geographically distant in respect of disease events. Detecting the spatial dependency would help researchers to justify the application of statistical models or smoothing techniques for disease mapping of rare disease or mapping is done for small boundaries. Spatial autocorrelation statistics provide a very useful summary information about the spatial arrangement of disease events in a map. Moran I and Geary`s C statistic are two commonly used spatial autocorrelation

statistics for detecting the global clustering in continuous areal data. There is also many spatial autocorrelation statistics available like Getis and Ord`s G, which measures the local clustering.

Mobile Phone Technology

Global spread of mobiles phones and networks at higher internet bandwidth, this technology is increasingly used to communicate early warning services and coordinate preparation activities. SMS alerts can be used to alert the local veterinary officers about the possible of occurrences of disease outbreaks. Advanced mobiles phones can also be used for video conferencing of disease information activities.

Early warning systems are combinations of tools and process embedded within institutional structures coordinated by national or international agencies. These systems are composed of four elements depending upon they focus on specific hazard or many, namely, knowledge of risk, a technical monitoring and warning services, dissemination of meaningful warnings to at-risk areas, and farmers awareness and preparedness to act. Warning services lie at the core of these systems, and how well they operate depends on having a sound scientific basis for predicting and forecasting. As early warning systems grow in geographical coverage and sophistication, false alarms to in rise. High false alarms can undermine the public confidence, breed mistrust, dilute the impact of alerts and reduce the credibility of future warnings.

National Animal Disease Referral Expert System (NADRES)

During 1987, The Indian Council of Agricultural Research (ICAR) established an All India Coordinated Research Project on Animal Disease Monitoring and Surveillance, (AICRP on ADMAS). On 1st April 2000, the AICRP on ADMAS was upgraded to Project Directorate on Animal Disease Monitoring and Surveillance (PD_ADMAS) (during IX Plan). The Directorate got further impetus with the addition of five more collaborating units in X plan and two mission mode NATP projects viz., Animal Health Information System and Data monitoring System (AHIS_DMS) and Weather based Animal Disease Forecasting (WB_ADF) having 17 and 20 collaborating units respectively. Combining the input from AHIS_DMS and WB_ADF, an interactive, dynamic online animal disease forewarning system called NADRES (National Animal Disease Referral Expert System) was developed with overall aim to improve the early warning and response capacity to animal disease threats in the country for the benefit of farmers. Presently NIVEDI is having 31 AICRP centers.

Early warning of disease incidence or outbreaks and the capacity of prediction of risk of spread to new areas is an essential pre-requisite for the effective containment and control of epidemic animal diseases, including zoonosis. Early warning is based on the concept that dealing with a disease epidemic in its early stages is easier and more economical than having to deal with it once it is wide spread. From the public health prospective, early warning of disease outbreaks with a known zoonotic potential will enable control measures that can prevent human morbidity and mortality. National Institute of Veterinary Epidemiology & Disease Informatics developed the software application, NADRES, which systematically collect, verify, analyze and respond to the information from designated AICRP-ADMAS, unofficial media reports and informal networks. NADRES builds on the added value combining the alert and response mechanisms of different organizations like state animal husbandry departments, Departments from universities, department of Animal husbandry, Dairying and Fisheries, AICRP on ADMAS and other agencies including NGOS, enhancing the capacity for the benefit of the farmers in the country and other stakeholders to assist in prediction, prevention and control of animal disease threats, including zoonosis, through sharing information, epidemiological analysis and joint missions to assess and control the outbreak, whenever needed. For Zoonotic disease events, alerts of animal outbreaks or incidence can provide the direct early warning so that human surveillance could be enhanced and preventive action can be taken. Similarly there may be cases where human surveillance is more sensitive and alerts of human cases precede known animal occurrence of disease. Sharing assessments of an outbreak will enable a joint and comprehensive analysis of the disease event and its possible consequences. Joint dissemination will furthermore allow harmonized communication by the Central and state Animal departments, ICAR-NIVEDI, regarding disease control strategies.

Risk factors data for NADRES

Data on risk factors was collected at village level as the village is the smallest unit at which key risk factors data such as livestock population, meteorological data and remote sensing variables. The data on livestock population in five major species viz., Cattle, buffalo, sheep, goat and Pigs were collected from 19^{th} livestock census at village level. Weather parameters from different sources includes the monthly precipitation(mm), sea level pressure (millibar),minimum temperature (^{0}C) maximum temperature(^{0}C) wind speed (m/s), vapour pressure (millibar), soil moisture(%) , perceptible water(mm), potential evaporation transpiration (mm), cloud

cover(okta) etc., extracted from National Centre for environmental prediction (NCEP), Indian Meteorological Department(IMD),National Innovations Climate Resilient Agriculture (NICRA) and other sources. The remote sensing variables like Normalized Difference vegetative index (NDVI) and Land Surface temperature (LST) were extracted from MODIS satellite images. Atmospherically corrected NDVI was collected on 16 day interval at 250 meter resolution using MODIS product MOD13Q1 and LST was collected on 8-day interval using MOD11A2 at 1 KM resolution. Soil profiles such as Soil pH and type of soil were collected at block level from ICAR-National Bureau of Soil sciences and Land Use pattern (NBSSLUP). All the risk factors are organized as raster (grid) type files and each predictor should be a raster representing a variable of Interest. These raster data typically stored in geo TIFF format.

Preventive Measures

Efforts to use environmental data for epidemic prediction and response began in the early 1920 in India. Utilizing of more than 10 years of metrological and remote sensing data and livestock health records (outbreaks), predicted risk maps are developed and these risk maps offer predictions 2-3 month lead time so as to allow government to response.Disease outbreak or incidence of notifiable diseases is detected in a specific region, it is important to prevent the spread of disease between animals, from animals to human and from humans to animals. This explains the how to use hygiene, biosecurity and farm health planning measures to prevent the spread of animal diseases. It is also includes the restrictions and other disease control measures such as protecting health workers, designating affected premises, setting up protection and surveillance zones, avoiding introduction of new animals, restrictions on sharing of farm equipments, preventing animals drinking from contaminated rivers and streams, farm visitors (people and vehicles) prevention of environmental contamination, control on livestock movements, vaccination etc.

References

- Laporte, R. E. (1993). How to improve monitoring and forecasting of disease patterns. BMJ: British Medical Journal, 307(6919), 1573.
- Wilson, M. L. (1994). Rift Valley Fever Virus Ecology and the Epidemiology of Disease Emergence a. Annals of the New York Academy of Sciences, 740(1), 169-180.

- Greenwood, B., & De Cock, K. (1998). New and resurgent infections: prediction detection and management of tomorrows epidemics.
- Rutherford, G. W., McFarland, W., Spindler, H., White, K., Patel, S. V., Aberle-Grasse, J &Stoneburner, R. L. (2010). Public health triangulation: approach and application to synthesizing data to understand national and local HIV epidemics. BMC Public Health, 10(1), 447.
- Rolka, H., Burkom, H., Cooper, G. F., Kulldorff, M., Madigan, D., & Wong, W. K. (2007). Issues in applied statistics for public health bioterrorism surveillance using multiple data streams: research needs. Statistics in Medicine, 26(8), 1834-1856.
- Lipsitch, M., Finelli, L., Heffernan, R. T., Leung, G. M., & Redd; for the 2009 H1N1 Surveillance Group, S. C. (2011). Improving the evidence base for decision making during a pandemic: the example of 2009 influenza A/H1N1. Biosecurity and bioterrorism: biodefense strategy, practice, and science, 9(2), 89-115.
- Ferguson, N. M., Cummings, D. A., Fraser, C., Cajka, J. C., Cooley, P. C., & Burke, D. S. (2006). Strategies for mitigating an influenza pandemic. Nature, 442(7101), 448.
- Mills, J. N., Gage, K. L., & Khan, A. S. (2010). Potential influence of climate change on vector-borne and zoonotic diseases: a review and proposed research plan. Environmental health perspectives, 118(11), 1507.
- Gale, P., Drew, T., Phipps, L. P., David, G., & Wooldridge, M. (2009). The effect of climate change on the occurrence and prevalence of livestock diseases in Great Britain: a review. Journal of applied microbiology, 106(5), 1409-1423.
- Thornton, P. K., van de Steeg, J., Notenbaert, A., & Herrero, M. (2009). The impacts of climate change on livestock and livestock systems in developing countries: A review of what we know and what we need to know. Agricultural Systems, 101(3), 113-127.

Emergence of Anthelmintic Resistance- constraint to livestock sector

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Introduction:

Helminth infestations are common in most animals but, particularly in adult healthy animals, theimmune system keeps the burden of helminths at such levels that clinical symptoms do not occur.However, if this balance is not obtained, which could be the case for young or diseased animals, orwhen infestation pressure is very high, helminth infestation may impact severely on the health statusof the animals. This may in turn affect performance and production (e.g. reducedmilk and weight gain) and could also lead to increased mortality.It is, therefore, important to ensure the availability of effective anthelmintics to treat animals. However, anthelmintic resistance (AR) in parasitic nematodes is a threat to sustainable livestock production worldwide and a growing concern for the control of parasites especially in the developing world. Our limited understanding of how resistance mutations arise and spread in parasitic nematode populations limits our ability to develop evidence-based mitigation strategies. Moreover, there is little information on the changes that occur in the genome as anthelmintic resistance mutations increase in frequency in parasite populations; the so called “genetic signature” of selection. Such knowledge is critical if we have to apply genome-wide population genomic approaches to identify new anthelmintic resistance mutations.

Anthelmintic resistance (AR) is defined as a decrease in the efficacy of an anthelmintic againsta population of parasites that is generally susceptible to that drug. According to Sangster, 1999, the development of AR in general is a very simple phenomenon and whatever the better way isused to control worms with drugs the more likely resistance develops. This is because drugtreatment leads to the survival of resistant worms, which, if allowed to reproduce, contributeresistance genes to the next generation. If the resistance genes have become fixed in theparasite genome (lack of genetic reversion) then AR becomes a prolonged phenomenon andthe susceptibility of parasites to the drugs to resume takes a long time. Less progress and lack of awareness to farmers to understand GIN infections and development of AR has been witnessed particularly in the developing countries.

Current status of AR

Although there is a lack of systematic monitoring data, the occurrence of resistance inmany helminth species against various classes of anthelmintics is evident through scattered reportsconcerning almost all domestic species. Several scientific reports indicateanincrease in helminth resistance to the older classes of anthelmintics (benzimidazoles,tetrahydropyrimidines, imidazothiazoles and macrocyclic lactones) (Borgsteede et al., 2007). Isolated reports on cases of helminth resistance to thenewest classes of anthelmintics have also been published (e.g. resistance of *Haemonchus contortus* tomonepantel, an amino-acetonitrile derivative, Van den Brom et al., 2015). Benzimidazoles are the oldest class of authorised anthelmintics; thiabendazole was introduced in the1960s. The first report of decreased efficacy of thiabendazole against *Haemonchus contortus* strainsdates from 1964, just 3 years after its introduction to the market (Van den Bossche et al., 1982).

Similarly, resistance has developed rapidly to other anthelmintic classes, particularly those used insheep and horses, after their introduction to the market. For example, resistance to imidazothiazoletetrahydropyrimidineandavermectin-milbemycinclassesdevelopedwithin3-9yearsafterintroductiontothemarketinsheep(Kaplan,2004).A major concern which is emerging is the decreased efficacy of triclabendazole against liverflukes (*Fasciola hepatica*) in sheep and cattle (Moll et al., 2000). Furthermore,resistant populations of *Cooperia* spp. to ivermectin are reported in cattle (El-Abdellati et al., 2010). Inhorses, resistance to benzimidazoles, pyrantel and macrocyclic lactones, has been reported forCyathostominae and *Parascaris equorum* (Geurden et al., 2014).In addition, *Oesophagostomum* spp. in pigs have been reported to be resistant to pyrantel (Roepstorffet al., 1987), levamisole and benzimidazoles (Gerwert et al., 2002), and resistance to pyrantel in ascarids and hookworm has been observed in dogs and cats (Riggio et al., 2013).

Mechanisms of resistance

Due to advances in molecular technology, mechanisms of resistance in worms are becomingincreasingly understood. The resistancein worms can be the result of a variety of mechanisms like geneticchanges in the drug target, changes in the drug transport (e.g. ATP-binding Cassette (ABC)transporters), or changes in the metabolism of the drug within the

parasite. Benzimidazole resistance in nematodes can be due to a mutation in thegene coding for the target site, the same mutation does not seem to cause resistance totriclabendazole in the trematode *Fasciola hepatica*. In addition, within a singleworm species, different mutations can lead to resistance to the same anthelmintic. For instance,benzimidazole resistance in *Haemonchus contortus* can be caused by the phenylalanine to tyrosinemutation at amino acid position 200 of the isotype 1β-tubulin gene (Kwa et al., 1994). However, thefrequency of this resistance point mutation (single nucleotide polymorphism, SNP) varies considerablyand it can be low in benzimidazole (BZ)-resistant populations which carry other mutations (e.g. codon 167). Although genetic selection contributes to resistance, changes in drug transport mechanisms or in the metabolism of the drug within a worm species also account for different resistance mechanisms to the same anthelmintic. The P-glycoprotein, a cell membrane transport protein able to transport many differentdrugs (including ivermectin, benzimidazoles and imidazothiazole derivatives), may lead to multi-drugresistance by increasing the active transport of drugs. Therefore, it can be concluded that more research is needed in order to understand themechanisms and to develop suitable assays for detection of resistance.

Methods of detecting resistance

There are various *in vivo* and *in vitro* methods available to assess the efficacy of anthelmintics.Furthermore, specific laboratory methods can be applied to confirm a suspicion of resistance in thefield, e.g. as described in the WAAVP study recommendations and guidelines .

Nematodes

Faecal egg count reduction test

Reduced efficacy, that may reflect the development of resistance, can be detected by using the FaecalEgg Count Reduction Test (FECRT). This test estimates the anthelmintic efficacy by comparing thenumber of worm eggs in faeces of infected animals before and after treatment. This test can be usedfor all anthelmintic classes, which is a great advantage compared to other tests. However, the sensitivity of the FECRT may be low. In addition, the egg output of somehelminth species varies depending on the density of the adult worm population. This is the case for*Ancylostoma caninum* in dogs or *Oesophagostomum dentatum* in pigs. In cattle, there is also no clear correlation between egg output and wormnumber. This illustrates that FECRT has limitations as atool for detecting resistance.

In general, FECRT can be used in horses, ruminants and pigs to detect nematodeswhich shed their eggs in the faeces. When evaluating the treatment effect, the interval betweentreatment and second sampling should be shorter than the pre-patent period of the specific worm.The correctsampling interval also depends on the type of anthelmintic, e.g. for persistent anthelmintics likemacrocyclic lactones the interval between treatment and faeces sampling is recommended to be 14–17days whereas for levamisole an interval of 3–7 days is advised. According to the WAAVP guideline on anthelmintic resistance egg count reduction of less than 90% (arithmetic mean) indicates resistance in pigs, provided that a minimum pre-treatment individual egg count was confirmed.

In horses, a reduction in FEC of less than 90% is suggested to indicate resistance but there is somedisagreement regarding this threshold. In some reports a mean of 95% is regarded to be anappropriate cut-off level (Larsen et al., 2011) whereas in other reports differentcut-off values for different classes of anthelmintics are proposed; e.g. 90% for pyrantel and 95% forbenzimidazoles and macrocyclic lactones (Dargatz et al., 2000). Further research is needed to concludeon the thresholds that indicate resistance when the FECRT is used in horses.

For small ruminants, according to the WAAVP guideline, resistance is confirmed when the percentageof reduction in egg count (arithmetic mean) is less than 95% and when the 95% confidence level isless than 90%. If only one of the two criteria is met, resistance is only suspected.

As previously mentioned, the usefulness of the FECRT as a tool to identify resistance is limited by itslack of sensitivity. Another disadvantage is that it is not species-specific; eggs of different nematodespecies cannot be differentiated within the test. Moreover, the interpretation of the test depends upon various factors including the detection limit of the method, the number of animals per group, the host species, and the level of egg excretion by the helminthes.

Egg reappearance period test

Egg reappearance period (ERP) is defined as the time interval between the last anthelmintic treatmentand the resumption of significant helminth egg shedding. To evaluate potentialoccurrence of resistance, the ERP after dosing should be compared with the historical ERP of theveterinary medicinal product.

Molecular assays

Molecular techniques, such as polymerase chain reaction (PCR) or pyrosequencing, can revealmutations in helminth genes responsible for resistance to a certain anthelmintic class. Currently, onlyresistance to benzimidazoles can be detected by PCR which is useful when resistance is caused by a single gene mutation (i.e. SNP), or by asmall number of such mutations.

Other methods

Other methods potentially useful for the detection of resistance are the egg hatch assay (EHA)and the microagar larval development assay (LDA). These methods havebeen developed for detection of resistance to benzimidazoles or levamisole in horses, pigs or smallruminants. Coles et al. (2006) have described these assays and how to interpret the results. Yetanother novel method for detection of drug resistant helminths is based on a digitalised evaluation ofworm motility. Maintained motility of nematodes after administration of an anthelmintic that shouldlead to paralysis of the parasite could indicate a lack of efficacy. This method has been described forlarval *Haemonchus contortus*, *Strongyloides ratti*, adult hookworms and blood flukes (Smout et al.,2010).

Trematodes and cestodes

Coles and Stafford (2001) proposed a "**dose and slaughter**" trial to substantiate suspectedresistance of trematodes in the field; after artificial infestation followed by treatment with a flukicide(e.g. triclabendazole), the animals are killed and the number of flukes in the liver are counted.The usefulness of the FECRT to reflect resistance has not been evaluated for tapeworms or flukes. Anegg hatch assay (EHA), recently developed for the detection of resistance against albendazole in*Fasciola hepatica*, needs to be validated (Robles-Perez et al., 2013). Fairweather et al. (2012) havedeveloped an EHA test for the detection of triclabendazole (TCBZ) resistance in *Fasciola hepatica* whichcould be useful.

Management strategies to delay the development of resistance

Different management strategies are used with the purpose of preventing infestation and/or keepinginfestation pressure low. This includes pasture management, refugia, and quarantine for animals which are newly introduced into a flock or herd. The overall aim is to reduce the need for anthelmintics andconsequently to delay the development of resistance. In

addition, when anthelmintic treatment isapplied, certain treatment practices are recommended with the purpose of reducing the risk forresistance development.

Correct use of anthelmintics

The prudent use recommendations currently established have the overall aim to target treatment inthe best possible way so as to reduce unnecessary exposure and thus limit the risk for resistance. Recommendations for prudent use of anthelmintics are generally based on an in-depth understandingof the helminth epidemiology. It is stressed that deworming is based on theconfirmation of worm burden and that treatment with a relevant product is applied at the right time inrelation to the life cycle of the parasite so as to obtain sufficient effect without unnecessary exposure. It has been demonstrated that underdosing and/or a too frequent use of anthelmintics belonging to thesame class will increase the risk for selection of resistance. It is recommended by experts that long-acting anthelmintics are applied only insituations when the grazing season is considerably longer than the duration of the effect (i.e. thatthese formulations are applied at the start of the grazing season).

The limited number of anthelmintics authorised for minor species leads to significant off-label use. Dosing strategies may then have insufficient scientificsupport which could cause, for example, unintentional under dosing. Although there is a lack of scientific evidence, rotation of anthelmintic classes is often recommended todelay the development of resistance. Rotation of drugs was originally suggested based on thehypothesis that reversion to susceptibility might occur if resistant worms were less fit than susceptible worms, and counter selection was applied via treatment with a drug from a distinct chemical class.

Routine deworming which is still often practised, leads to unnecessary treatment and, thus, anincrease of the selection pressure. This is of particular concern when used in farm animals in situationswhen environmental refugia (i.e. susceptible helminth population) is low. One example of routine useof anthelmintics on farms is the “dose -and- move” practice, which may provide a survival advantagefor resistant parasites.

It is generally agreed that the maintenance of refugia through the implementation of appropriatetreatment and pasture management routines is important to decrease the selection pressure andreduce the risk for resistance development (Graef et al., 2013).

Refugia

Resistance spread is promoted if parasites carrying mutations that bring about reduced susceptibility toanthelmintics are provided with a survival advantage in the population. The refugia concept aims tokeep the proportion of resistant worms within the population at a low level and it is thus advocated asa tool to slow the progress of anthelmintic resistance (Van Wyk, 2001). Parasites in refugia are thosethat have not been exposed to an anthelmintic, including those present as free-living stages in theenvironment, those in untreated individuals, and those in any lifecycle stages in the host that are notaffected by the anthelmintic treatment (Fleming et al., 2006, Van Wyk, 2001). Appropriate treatmentstrategies and pasture management need to be implemented to maintain refugia (Graef et al., 2013). Sargison has published an extensive overview of management measures to create refugia in sheephelminths (Sargison, 2011). The selective deworming of those animals that are predicted to be mostinfested by nematodes and/or to contribute most towards pasture contamination is implemented toslow the development of anthelmintic resistance but maintain a parasite population in refugia (Besier,2012, Sargison, 2011). In horses, the usefulness of this practice needs to be further scientificallyevaluated, however, the underlying principle remains the same (Nielsen et al., 2014). According to VanWyk (2001), the subpopulation of encysted equine cyathostomin larvae may be considered as a refugiapopulation as they escape the effect of anthelmintic treatment and reduce the selection for resistance through excretion of susceptible worm eggs onto pasture.

The value of maintaining a population of parasites in refugia to slow down the development ofanthelmintic resistance has been demonstrated in a bioeconomic model for sheep. In this model,besides the number of flock treatments, the proportion of the worm population in refugia had asignificant influence on the rate of development of anthelmintic resistance (Pech et al., 2009). Thesuccess of refugia (dilution) strategies relies on maintaining a sufficiently large susceptible populationof worms. To be successful, the early implementation of helminth control strategies according to therefugia concept is regarded necessary, i.e. acting when resistant allele frequency in the parasitepopulation is still low. It is also likely that implementation of control strategies according to thisconcept will be beneficial in regions or on individual farms where resistance is not currently a majorconcern.

Use of multiactive anthelmintic products

It is currently under discussion whether combination products that contain two or more activesubstances targeting the same helminth but through different mode of actions (so called multiactiveanthelmintic products) could be advantageous with respect to delaying the emergence of resistance.Modelling studies and some field data have indicated that such products may delay the development of resistance to new active substances (Learmount et al., 2012, Leathwick, 2012, Leathwick et al., 2012), or delay development of anthelmintic resistance to existing anthelmintic classes (Leathwick andHosking, 2009, Leathwick et al., 2015). However, the use of multiactive anthelmintics might select formultiple resistance to different anthelmintic classes (Wrigley et al., 2006, Leathwick and Besier, 2014,Geary et al., 2012). Whether multi-actives offer a benefit with regard to resistance development thatwould outweigh any risk for promoting multiple resistance needs to be further substantiated. This isimportant to determine before formulating any recommendation on the use of such products.

Other options

Other measures to control helminth infestation in animals are different pasture management routines,e.g. removal of faeces from pasture to reduce the level of infective larvae, reducing stocking densities,preventing high degree of infestation or improving drainage of pastures to decrease the risk of liver fluke infestations (Sargison, 2011). To be effective, such measures would have to be tailored accordingto the specific epidemiology situation on the individual farm. Knubben-Schweizer and Torgerson (2015)recommend that the farm epidemiological picture is determined by means of a detailed diagnosis of theaffected pasture and the group of animals before implementing appropriate measures against Fasciolahepatica in dairy cattle. Appropriate quarantine protocols are also recommended as a useful measureto prevent introduction of resistant helminths.

In addition to this, other biological control methods are currently under development, e.g. vaccines(Heckendorn et al., 2006, Waller et al., 2006, Hertzberg and Sager, 2006, Nisbet et al., 2016) and theselection for livestock that is genetically less susceptible to helminth infestation. The latter approachhas been tested in sheep (Stear et al., 2007).

Conclusions

Scattered information from different sources in Europe makes it clear that anthelmintic resistance ispresent all across the region, mostly in small ruminants. Currently, there are no EU-wide programmesthat systematically monitor the occurrence of resistance in helminths of relevant animal species. A fewlocal monitoring programmes for specific target species are running, but trends regarding thedevelopment of anthelmintic resistance in Europe are difficult to follow on the basis of such localprogrammes. Knowledge regarding the extent of resistance to different active substances in differenthelminth species in various geographical areas is useful for decision-making regarding theimplementation of activities aimed at controlling further spread. Therefore, a systematic monitoringprogramme in Europe would be of great value.

Demonstration of anthelmintic resistance is, however, difficult. For many helminth species, there iscurrently a lack of standardised/validated test systems for confirming resistance. More research isnecessary in order to understand the mechanisms and to develop validated methods that areaffordable and easy to use. The establishment of EU reference laboratories with the tasks ofmaintaining a reference strain library and evaluating and validating tools for monitoring anthelminticresistance could be very useful.

There is some scarcity of scientific data regarding important risk factors for resistance development ofhelminths in the different target species. Nevertheless, there is a common understanding amongexperts that measures to reduce the need of anthelmintics and promoting an appropriate use of thesedrugs are important to delay resistance development. Examples of prudent use advice are: to basetreatment on confirmation of worm burden or solid epidemiological information, to employ targetedselective treatment approaches at farm level and to avoid routine and frequent use, to dose correctlyand particularly avoid under-dosing, to use combination products only when all substances arenecessary for effective treatment, to manage pastures properly and to maintain an appropriate level ofrefugia, in particular by keeping a part of the herd untreated. Furthermore, although scientific supportis currently lacking, it is often recommended to rotate between different anthelmintic classes overtime. Particular care is necessary when pour-on formulations and prolonged release formulations areused with regard to the timing of administration and the management of animals after administrationof the product, i.e. to ensure sufficient drug exposure in all treated animals and to maintain a sufficientrefugia population.

A lack of narrow-spectrum products might result in unnecessary use of active substances, e.g.inappropriate use of fixed combination products. In addition, there is a current shortage of authorized anthelmintic products for minor species, which may unintentionally lead to inappropriate off-label use.

References:

- Borgsteede FHM, Dercksen DD, Huijbers R (2007): Doramectin and albendazol resistance in sheep in the Netherlands. Vet Parasitol., 144: 180-183.
- Coles GC, Jackson F, Pomroy WE, Prichard RK, Von Samson-Himmelstjerna G, Silvestre A, Taylor MA, Vercruysse J (2006): The detection of anthelmintic resistance in nematodes of veterinary importance. Vet Parasitol., 136: 167-185.
- Coles GC, Stafford KA (2001): Activity of oxyclozanide, nitroxynil, clorsulon and albendazole against adult triclabendazole resistant Fasciola hepatica. Vet. Rec., 148: 723-724.
- Dargatz DA, Traub-Dargatz JL and NC Sangster (2000): Antimicrobic and anthelmintic resistance. Vet. Clin. North Am. Equine Pract., 16: 515-536.
- El-Abdellati A, Geldhof P, Claerebout E, Vercruysse J, Charlier J. (2010): Monitoring macrocyclic lactone resistance in Cooperia oncophora on a Belgian cattle farm during four consecutive years. Vet. Parasitol., (1-2):167-171.
- Fairweather I, McShane DD, Shaw L, Ellison SE, O'Hagan NT, York EA, Trudgett A, Brennan GP (2012): Development of an egg hatch assay for the diagnosis of triclabendazole resistance in *Fasciola hepatica*: proof of concept. Vet Parasitol., 183:249-59.
- Gerwert S, Failing K and C Bauer (2002): Prevalence of levamisole and benzimidazole resistance in *Oesophagostomum* populations of pig-breeding farms in North Rhine-Westphalia, Germany. Parasitol. Res. 88: 63-68.
- Geurden T, van Doorn D, Claerebout E, Kooyman F, De Keersmaecker S, Vercruysse J, Besognet B, Vanimisetti B, Frangipane di Regalbono A, Beraldo P, Di Cesare A, Traversa D (2014): Decreased strongyle egg re-appearance period after treatment with ivermectin and moxidectin in horses in Belgium, Italy and The Netherlands. Vet. Parasitol., 204, (3–4), 291-296.

- Larsen, ML, Ritz, C, Petersen, SL, Nielsen, MK (2011): Determination of ivermectin efficacy against cyathostomins and *Parascaris equorum* on horse farms using selective therapy. Vet. J., 188, 44–47.
- Kaplan RM (2004): Drug resistance in nematodes of veterinary importance: a status report. Trends in parasitology, 20: 477-483.
- Kwa MS, Veenstra JG, Roos MH (1994): Benzimidazole resistance in *Haemonchus contortus* is correlated with a conserved mutation at amino acid 200 in beta-tubulin isotype 1. Mol. Biochem.Parasitol, 63: 299-303.
- Moll L; Gaasenbeek CPH; Vellema P and FHM Borgsteede (2000): Resistance of *Fasciola hepatica* against triclabendazole in cattle and sheep in The Netherlands. Veterinary Parasitology 91: 153–158.
- Riggio F, Mannella R, Ariti G and S Perrucci (2013): Intestinal and lung parasites in owned dogs and cats from central Italy. Vet. Parasitol. 193: 78-84.
- Robles-Perez D, Martinez-Perez JM, Rojo-Vazquez FA, Martinez-Valladares M (2013): The diagnosis of fasciolosis in faeces of sheep by means of a PCR and its application in the detection of anthelmintic resistance in sheep flocks naturally infected. Vet. parasitology, 197: 277-282.
- Roepstorff A, Bjorn, H and Nansen, P (1987): Resistance of *Oesophagostomum* spp. in pigs to pyrantel citrate. Vet. Parasitol., 24: 229-239.
- Smout MJ, Kotze AC, McCarthy JS, Loukas A. (2010): A novel high throughput assay for anthelmintic drug screening and resistance diagnosis by real-time monitoring of parasite motility. PLoS Negl Trop Dis. Nov 21; 4 (11), 139-151.
- Van den Bossche H, Rochette F, Horig C (1982): Mebendazole and related anthelmintics, Advances in Pharmacology and Chemotherapy, 19: 67-128.
- Van den Brom R, Moll L, Kappert C and P Vellema (2015): *Haemonchus contortus* resistance to monepantel in sheep. Vet. Parasitology 209: 278-280.