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Advances in diagnosis of livestock diseases

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Abstract

Livestock agriculture is carried out to produce food and fibre by the deliberate and controlled use of plants and animals. It is a manipulative ecology with its basic operational unit being the complex production system influenced by interactions, between biologic, climatic, economic, social and cultural factors. In recent years intensification has typified animal production. This trend has resulted in those diseases that manifest themselves primarily through a decrease in production efficiency and that, in most cases are endemic. The disease conditions often have a complex multifactor aetiology that is intimately related to the production system. Secondly, emphasis has shifted from the individual animal toward the population - a trend toward epidemiological considerations. The veterinarian and the farmers are interested in the frequency, distribution and determinants of health and disease (clinical or subclinical). Often times, productivity of the animal is used as a surrogate measure of health. Interest is in optimizing health as measured by productivity and not necessarily minimizing occurrence of disease. Diagnosis being the art of determining the nature of a case of disease and distinguishing that disease from another, has because of the uniqueness of livestock production and management, become very complicated requiring improvement in the methods for processing and interpretation of diagnostic tests. This has called for thorough understanding of the intricate relationship among animal characteristics, environmental factors, aetiological agent and disease in order to avoid misclassification of the population into healthy and diseased. The validity (Precision, specificity and sensitivity and cut-off-values) of a test are very crucial and evaluation of tests has become mandatory. There has, therefore, been developments in diagnosis ranging from clinical and/or ethinoveterinary medicine, agent isolation (direct or indirect) to epidemiological techniques. The catalogue of diagnostic tests includes microscopic examination (haematological or coprological) *in-vitro* tissue and cell culture; *in vivo* inoculation, xenodiagnosis and serological techniques (antibody and/or antigen detection). Antigen detection has been strongly influenced by modern biotechnology which has culminated into the polymerase chain reaction (PCR) and recombinant DNA and hybridoma technologies. Landscape epidemiology and nutrition status (trace element levels) have increasingly found a role in the diagnosis process, too. Disease in livestock comes about as a result of coincidence of many factors. Hence for diagnosis, there is need to integrate data from an array of procedures ranging from history, farming system characteristics to laboratory findings. It is the inference from the diagnostic tests rather than the observed test variable itself that is important.

Keywords: Epidemiology, diagnosis integration.

Background

Livestock agriculture is carried out to produce food and fibre by the deliberate and controlled use of plants and animals. The farmers keep livestock for many reasons. One reason stands out, however. To make more profit. Livestock production is manipulative ecology with its basic operational unit being the complex production system which is influenced by interactions between biologic, climatic, economic, social and cultural factors.

In recent times a number of trends have emerged in the field of livestock production, namely, intensification and increase in scale of operation. This practice has resulted in the occurrence of diseases that manifest themselves primarily through a decrease in production efficiency, and which, in most cases are endemic, becoming the most significant. Secondly, the farmers and veterinary emphasis has shifted from the individual animal in the herd toward the population (herd). This has accentuated the need for skills in quantitative methods. Epidemiology being the study of the frequency, distribution and determinants of health and disease in a population has of necessity become a key discipline in livestock health and production. Although health in human has been defined as a state of complete physical, mental and spiritual well being, in veterinary medicine productivity is often used as a surrogate measure of health (Moss 1992 and Martin et al. 1987). Whether the disease is present or not is usually less important than the frequency with which the disease occurs and its subsequent impact on productivity. In any case disease may not be the most important limiting factor of production; other factors (housing, feeding), may. Epidemiology is therefore the population analogue to the pathogenesis of disease in an individual.

So in the changing livestock production system, decisions regarding animal health can rarely be made solely on biologic grounds. Rather a dynamic integrated approach combining epidemiologic and economic analyses is required to determine the nature and scope of the health problem and implications of any intervention. Whether

the animals have a particular health problem is largely immaterial unless it is economically advantageous to do something about it. Zoonotic diseases are exceptions to this caution, however.

Diagnosis is the art of determining the nature of a case of disease and that of distinguishing one disease from another. This is done through use of diagnostic tests which basically are devices or processes designed to detect/elicit a sign, substances, tissue changes or response. Such substances include the presence of causative agents or their products or specific antibodies in serum or the plasma concentration of an enzyme. Other features can be investigated without laboratory facilities (e.g. clinical examination and skin reaction after tuberculin test). As regards livestock health and production, both diseased and healthy animals are studied through epidemiological investigations. Such investigations are the diagnostic tool for populations analogous to the role of clinical medicine as the diagnostic tool for individuals. Individual animals are observed but the conclusions are based on the experience of the group (herd). The function of the test is to help decide the disease status of the animals. Data on which to base a rational decision for prevention or control of disease in the animal population has to be provided. In livestock this commonly involves optimizing health (productivity) and not necessarily minimizing occurrence of disease.

To fulfill these purposes, investigations might be carried out to (i) describe the disease: what is the disease syndrome? estimate the frequency of the disease, who has it and where is it? and when did it occur? (ii) analyse and identify factors that might cause the disease and is it endemically stable or not, namely, test hypotheses and answer the question "why"the disease occurred?

The working definition of what constitute a 'diseased herd' is determined by the importance of the disease. For extremely damaging diseases like New Castle Disease (NCD) a finding of one infected animal results in the entire herd or region being declared infected. We also have situations where we accept to "live with" infection in that we accept levels of infection below a certain threshold. For example, *Staphylococcus aureus* mastitis is endemic in many herds.

Advances

The advances in the diagnosis of livestock diseases have, therefore, included improvement in the methods for processing and interpretation of test results. This has called for thorough understanding of the relationship among host characteristics, environmental factors, aetiological agents and disease (or reduced productivity).

The existence of measurement error in diagnostic tests is recognized and everything should be done to minimize the error lest one draws incorrect or inconsistent conclusions. Secondly, it is desired that the test result should correlate with the disease status of the herd although this is not always achieved. Test evaluation to assess the performance of a diagnostic test is very important. Inferences from the results of any diagnostic test are only valid when the test was evaluated carefully before being recommended for use. The consequences of false-positive and false negative test results are often associated with costs, losses and physical discomfort. Therefore the risk of false test results must be known. This is because a characteristic of diagnostic tests is that the inferences from the test results rather than the observed test variable itself are of medical importance. Therefore the validity of a test, that is, precision (repeatability) and accuracy (ability of a test to give correct results as measured by sensitivity and specificity) is very crucial in interpretation. Sensitivity (SEi) is test positive animals divided by truly diseased animals while specificity (SPi) is test negative animals over the truly non-diseased animals. Depending on whether one emphasizes sensitivity or specificity one can determine cut-off values. A high-cut off value is normally associated with increasing specificity. That is, false positive cases are not acceptable while false negative cases are acceptable. This way, misclassification between healthy and diseased is avoided. This is common in surveys with emphasis on conservative estimate of seroprevalence. Low cut off values, on the other hand, lead to an increase in sensitivity. This time false negative cases are not acceptable while false positive ones are acceptable. The consequences of false positive result are not severe. Yet, a false negative result has a negative impact in terms of costs and welfare. This is commonly the case in surveillance and screening exercises (e.g Rinderpest surveillance).

Other parameters of evaluation include economic evaluation, namely the cost/benefit ratio; and operational analysis.

The tools of diagnosis encompass clinical examinations, laboratory techniques, and shute-side techniques (for isolation and identification of aetiological agents) and farming system investigation including history and landscape epidemiology. Clinical and ethinoveterinary procedures have constituted the first line in diagnosis. The procedures may be invasive or noninvasive and the techniques can be qualitative or quantitative or semiqualitative. The test can be pathognomonic (an absolute predictor of the presence of the disease or disease agent) or surrogate (detect secondary changes which are hoped to predict the presence or absence of disease or the disease agent).

The techniques for isolation and identification of aetiological agents can be classified as direct or indirect. The direct ones include microscopic examination of specimen, *in vitro* tissue and cell culture, *in vitro* inoculation and Xenodiagnosis. Direct techniques are, most reliable and definitive. However, they are less sensitive, they are protracted and not ideal for large scale screening. To overcome these problems, indirect methods have been developed. These are based on measurement of some variables and serological detection of antibodies. These include Indirect Hemagglutination (IHA), latex agglutination (LA), Complement fixation test (CFT), Indirect fluorescent antibody test (IFAT) Enzyme Linked Immunosorbent Assay (ELISA) and Western blot.

The two handy techniques for evaluating anaemia are: (i) the Packed Cell Volume (PCV) or the haematocrit and (ii) the blood smear. The PCV is the best single procedure

for evaluating the condition of the erythron and the degree of anaemia. One needs a microhaematocrit centrifuge and haematocrit reader. This technique is very useful in nagana cases (Woo 1970). The normal PCV in cattle is 30 - 35% but in trypanosomiasis it is 12 - 24%. When the PCV drops to 28% you have a problem. In addition one can look at the PCV categories. In nagana inflicted herds one gets "PCV Profile Shift" to the left because of anaemia.

The blood smear is indispensable procedure. Hypochromasia and increased erythrocyte production (reticulocytosis) is best seen in regenerative anaemia when the smear is stained with new methylene blue. For blood parasites Giemsa stain is satisfactory. These direct methods are very sensitive, specific, simple and cost effective and hence can be applied in screening. However, these techniques, are not definitive and hence not ideal indicators for chemotherapeutic intervention. They are, on the other hand, important for epidemiological investigations.

(i) The IFAT depends upon a reaction between a particular antigen (fixed onto defined areas of commercially available slides) and immune serum. The globulins of the latter are made visible under ultra-violate light after reaction with fluorescein - labelled anti-species serum (conjugate). It is a routine serological test in the diagnosis of *Theileria* species. Using this test, cross-reactions are observed between *T.parva, T.mutans, T.annulata* and *T.taurotragi* (Burridge et al 1974, Grotenhuis et al 1979). Using appropriate controls, the test can be useful in identifying these species. Monoclonal antibodies (MAbs) raised against the macroschizont stage of the parasites have been used successfully to detect antigenic diversity of *T.parva.* The principle is that different strains of *Theileria* parasites possess strain specific antigens that are difficult to study directly. MAbs have been produced to identify these parasite strains. Firstly, *Theileria* parasites material is injected into mice to stimulate antibody production. Then the antibody producing spleen cells are taken from the mice and fused with myolemma (bone marrow) cells which can grow and multiply in culture. The resulting hybrid cells are cloned and appropriate clones are selected to produce MAbs. Twenty two stocks of *T.p.parva* have been isolated from widely separated areas and screened using a battery of 16 MAbs. The responses of the different antibodies are measured by IFAT. This way stocks from Uganda, Malawi, Rwanda, Kenya and Zambia have been characterized and the diversity has been found to be small. Four predominant groups (A, B, C, D) have emerged. It is too early to say whether the four groups of parasite can be considered as four strains, but nevertheless, the finding that antigenic diversity is small with *T.parva*, gives hope that the number of strains is also small and that immunization of cattle with one or two key strains may protect them against ECF throughout the range. However, IFAT limitations in the field must be recognized especially in large areas of eastern, central and southern Africa where *T.parva T.taurotragi* and *T.mutans* occur concurrently.

(ii) The ELISA: The antigen is attached to the plastic wells. The antibodies in the test serum when present attach to the antigen. The retained antibody is detected by an anti-species serum conjugated to an enzyme (mostly alkaline phosphatase) which in the presence of a suitable substrate produces a colour reaction, the intensity of which is proportional to the antibody titre.

(iii) Immunoblotting (Western Blot) is similar to the ELISA in principle: Antigen samples are separated in an analytical gel (SDS polyacrylamide gel). The resolved molecules are transferred electrophoretically to a nitrocellulose membrane in a blotting tank. The blot is then treated sequentially with antibody to the specific antigen, and washed and then a radiolabelled conjugate to detect antibody is a bound to the blot. After washing again, the blot is placed in contact with an X-ray film in a cassette for autoradiography. The autoradiograph is developed and the antigen bands which have bound the antibody are visible.

(iv) Complement Fixation Test (CFT): Complement (a system comprising of at least 20 distinct serum proteins that is the effector of immune cytolysis and other biological functions) is fixed during the reaction between some antigens and antibodies. By conducting this reaction in the presence of measured amounts of complement and then detecting the remaining unfixed amount with a separate antigen-antibody reaction, a quantification of the complement fixing activity of the original serum is obtained. More and more, CFT is being replaced by IFA and ELISA.

The limitations of the indirect techniques based on antibody detection have been overcome by techniques which detect antigens or the antigen's nucleic acids (DNA and or RNA). Presence of antigen is ample evidence for an on-going infection and thus antigen detection is an important approach to immunodiagnosis. Antigens can be detected in faeces, biopsies or blood or other fluids by using monoclonal antibodies. Antigen-aemia due to toxoplasmosis, hydatidosis, and trypanosomiasis (Nnatulya and Lindquist 1989; and Nnatulya et al 1989) can be detected by ELISA and LA. Initial reports indicate that this test is considerably more sensitive than trypanosome identification procedures and is specific for infections with *T.bruce, T.congolense and T.vivax.* However, field evaluations of this test for *T.vivax* have been inconsistent (Peter Vanden Bossche personal communication).

Antigen detection has been strongly influenced by modern biotechnology culminating into polymerase chain reaction (PCR), and recombinant DNA and hybridoma technologies.

(a) The PCR is an *in vitro* method for enzymatic synthesis of specific DNA sequences using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA.

Despite the fact that species-specific probes tend to detect highly repeated sequences in the genome (Vide infra), the limits of detection are still in the nanogram range (10^3 - 10^4 oocysts). The PCR has opened up extensive possibilities for the detection of the aetiological agents.

The PCR requires a special equipment (a thermocycler) and the PCR reaction mixture consists of the DNA sample to be tested, two specific oligonucleotides (primers), a temperature resistant DNA polymerase and a mixture of nucleotides which form the building blocks for DNA synthesis. The oligonucleotide primers are designed to match and bind at two well-defined sites of a known parasite DNA sequence. The primer is capable of binding to a sequence containing matching complementary bases.

The double-stranded DNA in the sample is first made single stranded (Melted) by incubation at high temperatures (95EC). The reaction is then cooled down to a lower temperature (55EC) at which the primers can bind the specific target sequences in the DNA sample. The temperature is then raised again to 72EC at which the polymerase synthesizes two new strands of DNA using the specific primers as starting point and the target DNA fragments as a template. The three events, melting, primer annealing and DNA synthesis constitute one cycle. By continuously repeating this cycle, the polymerase synthesizes increasing numbers of copies of the target sequence and the fragment is thus "amplified" so that it can be detected by agarose gel electrophoresis. Starting with one molecule, 10^9 molecule can theoretically he generated in 30 cycles. As little as 10 picograms of parasite DNA are sufficient to detect the parasite i.e less than 1000 oocysts of *Eimeria* can be detected.

(b) The detection of infectious disease pathogens and identification of genetic variation associated with disease has been revolutionized by the use of specific nucleic acid hybridization probes. A probe is a molecule having a strong interaction only with a specific target and having a means of being detected following the interaction. The genetic material (genome) of parasites that are considered distinct species show significant differences which one could call their "genomic signature", allowing them to be distinguished from each other. Technologies now exist that enable the isolation and identification of such specific sequences resulting into the generation of DNA probes. Such probes can be long consisting several thousands of nucleotides (a base + a sugar which could be ribose or deoxyribose + a phosphate group] or short (20 - 30 nucleotides). In order to allow detection, parasite (agent) DNA needs to be immobilised on a nitrocellulose or nylon membrane. Alkaline denaturation causes the separation of the DNA strands, making the sequences accessible and allowing the DNA probe to bind the target sequence by a process called hybridization. Binding of the probe to parasite sequence can be demonstrated by labelling it before hybridization. Radioactivity labelled nucleic acid probes have been in wide use for two decades. However, DNA probes modified with non-radioactive labels are revolutionizing molecular biology and clinical diagnostics. Colour reactions or chemiluminescence are used to demonstrate specific hybridization. They are several ways in which parasite nucleic acids can be transferred to membranes. Droplets of fluid to be tested for the presence of the aetiological agent (blood, lymph other fluids or stool) can simply be applied to membranes and left to dry. This is the "dot - blot" process. Parasite containing vectors (infected tsetse, ticks or snails) can also be squashed onto membranes. This is the "squash blots".

So with a minimum of equipment DNA probes could be used under the most primitive field-laboratory conditions. Membranes on which material has been blotted can be dried and stored for a long time before analysis.

Another advantage of the techniques that depend on detecting genomic sequences is that whereas the morphology and the antigenic repertoire of the different life-cycle stages of a parasite can vary extensively, the parasite genome is very stable. Species specific probes can thus help to circumvent problems associated with identification of parasites by light microscopy. Most samples can be stored indefinitely in 70% ethanol/1M EDTA and DNA can easily be analysed at a later point by the "Southern blot" hybridization. This involves the extraction of DNA from samples, digestion with a number of chosen restriction enzymes (enzymes that cut specific sequences of DNA), separation of the DNA fragments by electrophoresis, transfer by capillary blotting to a membrane and hybridization with specific probes. The same technique performed with RNA is called "Northern blotting".

It is important to realize that the of specific probes or specific primers for PCR implies that they only provide a positive identification of the parasite for which the probe/primer were designed. PCR has helped realize the potential of DNA based diagnosis by producing enough of the target sequence so that simple rapid and robust methods for identifying the sequence could be employed.

In many diagnostic tests the detection of anti-parasite (agent) antibodies in the serum of infected/immune animals depends on the availability of specific antigens that have to be purified from the organism. This is usually difficult and expensive. Using the technique of gene cloning, it is now possible to isolate parasite genes encoding a single antigen and introduce these genes into host cells (bacteria, yeast or mammalian) where they can be expressed in large quantities as a recombinant antigen. It is also possible to "engineer" these antigens and remove those parts that are responsible for cross-reactivity with other organisms. This way, a continuous and reliable source of diagnostic antigen is guaranteed and increased specificity can also be achieved.

Recombinant DNA technique create molecular clones by conferring on a specific sequence the ability to replicate by inserting it into a `vector` and introducing the `vector` into a host cell.

A `vector` is a plasmid (minichromosomes or genetic elements that are not linked to the main chromosomes) or viral chromosome into whose genome a fragment of foreign DNA is inserted and is used to introduce the foreign DNA into a host cell in the cloning of DNA. Recombinant technology has potential in producing viral proteins of the foot - and - mouth - disease virus (FMDV) of cattle. The large vaccine market for this disease is presently met with conventional vaccines of inactivated particles. Viral proteins from recombinant DNAs should be less expensive than propagating the virus in animal cells and might lead to safer and cheaper vaccines.

Conclusion

In conclusion, a microscope, a well-trained eye and the application of immunodiagnostic tools adapted for the field will, by and large, cover the needs of the person in the field involved in routine diagnosis of livestock diseases and other problems. The field workers should function as an important interface between the field and research laboratories. However, epidemiological investigations are often called for and it is especially in this area that molecular biology techniques have brought sensitivity and specificity to a level where a single parasite can be detected. Molecular cloning and the production of recombinant proteins has led to the expansion of the range of parasites for which diagnostic antigens can be produced.

The older concepts about aetiology and pathogenesis of disease have had to be restructured. It is now realized that the aetiological agent (parasites, bacteria or viruses) is only a single participant in the complex of causality of disease. The search for aetiology of disease has been replaced by the search for patterns or a network of changes within which the disease states occur. We are talking of a "web of causation". Disease comes about as a result of coincidence of many different factors. Disease is a set of manifestation of the host - agent - environment interaction. Disease, therefore, exists in "nests" (nidi). Hence there is need to integrate data from an array of procedures ranging from history, farming system observation (landscape epidemiology and management regimes) to laboratory findings (parasitological, immunodiagnostic and molecular biological) before one comes to accurate or/helpful diagnosis. Commonly it is the inference from the diagnostic tests results (e.g. shifting to the left of haematocrit (PCV) values in nagana and endemic stability (of tick-borne diseases) rather than the observed test variable itself (PCV and high seroprevalence) that matters. PCV values have to be looked at in association with the distance the herds are from the tsetse foci. So has the high seroprevalence got to be associated with the demographic structure of the herds and the presence or absence of the tick vectors. Yes, there have been advances in the diagnosis of livestock diseases. But because of the complementarity of the diagnostic tests, none of them is out-dated and discarded todate. All one needs to be aware of, is their individual limitations and advantages.

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