Present and pending diagnostic procedures for determining disease agents on zona-intact embryos*

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Summary: Embryo transfer can lead to the transmission of disease agents. The characteristics of each infection will determine the choice of the most sensitive and cheapest diagnostic methods for routine use.

Various diagnostic procedures which are already in existence or are being developed according to advances in biotechnology, are reserved for experimental work.

According to the disease agent sought in the embryo's environment, samples are taken from three sources: 1) the donor cow, 2) the flush fluids and the fluids from the washings of embryos, and 3) the embryo (or a representative sample including degenerate or non transferable embryos). Because freezing and thawing of embryos may inactivate a virus, it is advisable to analyse them within 24 hours of collection.

Ultracentrifugation enables the isolation of a virus from extended semen.

Specific test methods are presented and discussed for six diseases: bovine leukaemia, infectious bovine rhinotracheitis, bluetongue, bovine viral diarrhoea, vesicular stomatitis, and Brucella abortus infection.

In the discussion, the author is of the opinion that negative results should be viewed with caution since they call into question test sensitivity and specificity, appropriateness of sample size or the technical competence of the testing laboratory.

Methods other than trypsinisation are envisaged to dislodge a virus from the zona pellucida.

KEY-WORDS: Bluetongue virus - Bovine diarrhoea virus - Brucella abortus - Cow - Diagnostic techniques - Disease control - Embryo - IBR/IPV virus - Leukaemia virus - Transplantation - Vesicular stomatitis virus.

INTRODUCTION

Sanitary problems related to embryo transfer must be fully resolved in order that this new technology can play a determining role in the future movement and distribution of superior genetic stock to various parts of the world. Based on the publish-

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ed literature regarding embryo transfer, this new technology promises excellent potential to provide a means for the rapid, economical and safe distribution of valuable genetic traits. However, the adoption of control methods that would fail to prevent disease transmission might not only jeopardize the world livestock population but, in the final analysis, could be as detrimental to the embryo transfer industry as controls that are exceedingly and unnecessarily stringent and would hamper or stifle development of trade in embryos. The responsibility entailed in taking decisions regarding these matters is evidently of the utmost importance.

Firstly it would be appropriate to state some fundamental concepts on the subject of diagnostic procedures.

1. There are no universal test methods or systems. Each infectious entity will ultimately have to be considered on the basis of its own individual characteristics and test systems will have to be developed accordingly.

2. There is no such thing as a perfect test. One can readily envision instances in which serological or immunological tests would fail because the infection is so recent that responses have not developed. Similarly, tests for etiological agents may fail because the agents are in an eclipse phase. There is also the possibility that the infectious agents are present in such small numbers that sample size limitations preclude isolation and identification.

3. There can be no totally safe approach to embryo movement, or the movement of live animals, semen or other animal products for that matter. This would be attainable only by the complete prohibition of movement. We are, therefore, engaged in a process of discussing methods to minimize risk.

**GENERAL TEST METHODS**

The application of specific methods or test systems will not only vary with each agent but, obviously, some methods will only be possible or applicable for research studies rather than for routine diagnostic purposes. Thus, the transfer of embryos from infected or potentially infected dams into susceptible recipients would seem to be the single most relevant method for accurately determining the potential for disease transmission. This is, however, obviously not a diagnostic technique and even in research situations is such a complex, costly and time-consuming procedure that the accumulation of statistically significant numbers of test observations may be difficult. In some systems (bovine leukaemia virus and bluetongue virus), the inoculation of test materials into susceptible sheep or cattle may provide the most sensitive and accurate test for research purposes but may be too costly, cumbersome or time-consuming to be of practical value in routine diagnosis. These aspects will be further discussed in relation to some of the specific disease agents. Continuing advances in monoclonal antibody techniques and biotechnology have resulted in the development of nucleic acid probes which can identify viral agents in their replicative states, or antigen capture and enzyme histochemical procedures that can identify extremely small quantities of microbial proteins. Most of these tests are, however, still in various developmental phases and have not been extensively validated in comparisons with virus isolation results. Thus, although they may in time supplant current microbiological test methods, evidence that they will detect less than one infectious unit of viral pathogens is as yet unverified. For this reason, and because infectivity is, in fact, the criterion of most importance, these methods will not be extensively discussed in this report. They are of the most value, at their
current state of development, for the specific identification and typing of organisms that have already been isolated by conventional methods because most of these techniques still require the amplification of the viral pathogen by *in vitro* replication before they can be utilized.

Sample selection is an additional and important aspect in any discussion of test systems. For the purposes of this discussion three sample sources will be considered: 1) The embryo donor because there are a few instances in which information can be gained more accurately and more readily from samples obtained from this source. 2) The flush fluids and the fluids from subsequent washings of embryos because they provide specific information regarding the presence of potential contaminating pathogens in the immediate environment of the embryo. 3) The embryo (or rather a representative sample of these, including degenerate, non-fertile and non-transferable zona-intact embryos) because they are the specific entity to be involved in international commerce. It is difficult to envisage situations in which embryos without the zona pellucida intact could be successfully handled in most of the proposed washing procedures, so testing of embryos of this type would not have any value. Samples from the sire or semen have not been included because there is little evidence that infection of the embryo itself originates from this source. If semen contains infectious material, and actually establishes a genital infection in the donor, this should be identified in tests of the flush fluids.

The processing and handling of test specimens is also of considerable importance. Because freezing and thawing may inactivate fragile agents, and would seriously decrease chances of isolation of cell associated or integrated viral agents, the rapid delivery of refrigerated, but not frozen, samples to the diagnostic laboratory would be preferred. If storage or prolonged transit is necessary the specimens would, of course, have to be frozen. Preliminary tests in our laboratory using flush fluids intentionally contaminated with IBR virus would suggest that large volume samples could be concentrated by ultracentrifugation and the pellet tested for infectivity. This technique has been found to be useful in attempts to isolate viruses from extended semen (1). If volumes of wash fluids were so large that inoculation of the total volume would damage the cell culture system, these samples should also be concentrated by ultracentrifugation. The sonication of embryos to dislodge or dissociate viral agents has been useful in studies of susceptibility to viral infection and is recommended. An additional approach to testing intact embryos would be to expose them to the test cells in suspension, perhaps including a low speed centrifugation to pack the tissue culture suspension around the embryos to increase the chances of contact between the entire surface of the embryo and the test cells, with subsequent plating and culture of the test cell suspension.

**SPECIFIC TEST METHODS**

The agents to be discussed were chosen because they have been considered to be of importance in international trade, because they have been investigated to some extent so information is available from the literature, and because they illustrate some of the diversity encountered in attempting to select appropriate test methods. The list is therefore intended to be illustrative in nature rather than specific or complete.
BOVINE LEUKAEMIA VIRUS

General information

Because retroviruses have the capacity to insert DNA transcripts of their RNA genome into host chromosomes, some of these viruses have become endogenous to their host and are now transmitted from generation to generation as a host gene. BLV is not carried in this manner but is horizontally transmitted in the same manner as other infectious agents (2). Serological responses, and lymphocytes carrying the BLV genome, persist in the blood of infected cattle for life. Thus, infectivity is usually present in flush fluids from BLV positive donors because of the contamination of flush fluids with blood. There is no evidence that the transfer of embryos from BLV infected donors causes seroconversion or infection of recipient females or results in the birth of BLV infected calves (3, 4).

Suggested test methods

Because BLV seroconversions and persistence of virus in the lymphocytes are virtually synonymous and the in vitro syncytium induction assays for infectivity are cumbersome and rather insensitive, serological testing of donors would provide the simplest and best method to evaluate the potential for virus exposure of embryos in the initial flush fluids. Flush fluids, wash fluids and embryos could be assayed by syncytium induction assays but radioimmunoassays or antigen capture techniques, after in vitro culture of the test material, would probably be as sensitive and would eliminate problems due to the possible presence of other syncytium inducing viruses in the donor cattle. The most sensitive infectivity assay would involve the inoculation of test sheep but this method would probably only be applied in research, not diagnostic, situations.

A potentially useful test in this situation would be a sensitive assay for haemoglobin. Because the virus is blood-borne, evidence that washing had been sufficient to remove erythrocytes and haemoglobin would provide indirect evidence that potential carriers of BLV were also removed.

INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS

General information

IBR infections have a particular affinity for respiratory, reproductive and adrenal tissues. Infection results in seroconversion, but virus is not completely eliminated from the animal and can be recrudesced, probably from neural ganglia associated with lesion sites, by treatment with corticosteroids or "natural" stress. Presence of detectable antibody indicates either recovery from infection or previous vaccination and the two responses are not differentiated by commonly applied tests. A positive serological status is usually associated with a negative virological status but, as indicated above, viral reactivation is possible so, rarely, it would be associated with a positive virological status. The absence of antibody suggests no previous exposure to the virus or, rarely, an acute infection. Reactivated virus has been recovered from both nasal and vaginal swabs (5). In studies in our laboratory, where cattle were slaughtered after dexamethasone treatment, virus was not recovered from the ovary, uterus or blood, but was found in the corpus luteum of 1 in 6 heifers (6). In a subsequent unpublished study we isolated IBR virus from the adrenal.
ovarian tissue, oviduct and infundibula of a heifer that had been inoculated 7 days after breeding and treated with dexamethasone 5 months later.

The transfer of washed, trypsin treated, embryos collected from IBR infected donors, even during the acute phase of infection when virus was present in uterine flush fluids, did not result in transmission of IBR to recipient heifers. Other studies indicate, however, that IBR virus adheres to the zona pellucida and is not always removed by washing in the absence of trypsin treatment (7, 8).

Suggested test methods

Because the serological tests do not accurately differentiate among infected, recovered and vaccinated animals they are of limited value in accurately assessing the virological status of donors. IBR virus grows readily in a variety of cell culture systems and is markedly cytopathic so virus isolations from flush fluids, wash fluids and embryos would provide the most accurate means of differentiating IBR contaminated and non-contaminated embryos. As indicated above, because of the tendency of IBR virus to stick to the surface of the zona pellucida, special handling (trypsin treatment) is needed to be certain that contaminating viruses have been removed by washing.

BLUETONGUE VIRUS

General information

There are few viral infections of cattle that have stimulated more controversy than bluetongue virus. There have been substantial disagreements as to whether the virus is in fact a bovine pathogen, disagreements as to the sensitivity and validity of various serological and virological test methods, and conflicting reports regarding the existence of persistent bluetongue infections in cattle. Although the advent of more sensitive serological methods has somewhat clarified the situation regarding the seropositive to seronegative fluctuations reported previously, there is still some uncertainty associated with the interpretation of serological test results. A somewhat similar situation exists with the virological test systems. Results of one comparison of cell culture, embryonated egg and sheep inoculation failed to identify a superior system because, in individual cases, each of the test methods seemed to be best. This led the authors to conclude that a multiplicity of systems should be used if maximum sensitivity is desired (9). There are also apparent fluctuations in the level of viraemia in persistently infected cattle and a similar situation exists regarding the presence of virus in the semen of infected bulls, with an apparent intermittent appearance of virus. Inseminations of donor cows with bluetongue virus infected semen or experimental inoculations of bluetongue virus into donor cattle have resulted in the identification of virus in flush fluids but the transfer of washed embryos from these cattle has not resulted in the infection of bluetongue virus-negative recipients (10, 11). Exposure of embryos to bluetongue virus in vitro has not resulted in embryo infection or death (12).

Suggested test methods

As indicated above, the serological tests of donor animals provide only very limited information regarding the virological status of the embryos. Virological tests, including cell culture (Vero cells), intravenous inoculation of embryonated
chicken eggs and sheep inoculation of flush fluids, wash fluids and embryos would provide the most valid information regarding the virological status of the embryos destined for transfer. Because the virus is often blood cell associated, non-specific tests for blood or haemoglobin in the final wash fluids, as suggested for BLV, would provide useful information regarding the efficacy of the washing procedures.

BOVINE VIRAL DIARRHOEA VIRUS

General information

BVDV is associated with two distinct clinical entities in cattle. One is an acute febrile enteric condition associated with diarrhoea and low mortality and the development of a positive serological response that is associated with a clearing of virus, thus making it difficult to isolate virus from these individuals. A second entity, mucosal disease, is, on the basis of recent information, associated with a relatively long prodromal state during which there is a persistent viraemia in the absence of detectable antibody production. This is followed by the development of a severe erosive condition involving much of the digestive tract accompanied by a marked depletion of lymphoid tissues. The mortality rate is high. The initiation of the pathological condition is presently considered to be the result of the superinfection of the persistently infected animal with a different (cytopathic) BVDV (13).

Although there has been one report in which virus-like particles were identified in the embryo, but not infectivity (14), it seems likely that BVDV does not penetrate or adhere to the zona pellucida (12, 15).

Suggested test methods

Because of the existence of persistently infected seronegative animals, serological testing of the dam would be of limited value in evaluating the potential for BVD transmission by embryo transfer. In fact, in this instance, a seropositive response by the donor may even be desirable. Virus isolation in cell culture would be the method of choice for identification of virus in flush fluids, wash fluids and embryos. Because many isolates are non-cytopathic, immunofluorescence or a similar immunoenzyme technique will be required for the evaluation of the test cell culture monolayers. Potential problems due to the use of flush or wash fluids contaminated with BVDV from serum or other components should be recognized.

VESICULAR STOMATITIS VIRUS

General information

VSV causes an acute vesicular disease, usually of limited duration. Seroconversion is permanent. Recurrence of lesions has been observed in animals transported 4-6 weeks after the initial lesions healed, but long-term studies indicate that there is no reactivation of virus. We have been unable to reactivate virus by treatment with dexamethasone (16). Viraemia is brief or absent. The intravenous and intradermal lingual inoculation of virus into two heifers on the day after breeding did not result in the presence of infectivity in the genital tract specimens collected from these animals at necropsy thirteen days later (16). A recent report indicates that, after in vitro exposure, VSV adheres to the intact zona pellucida (17).
Suggested test methods

In this instance the use of serological tests would yield almost universally false information regarding the virological status of the embryos. Virus isolation in cell culture would be the most useful test.

BRUCELLA ABORTUS

General information

Infection results in seroconversion which cannot be accurately differentiated from seroconversion due to vaccination. In cases of abortion, organisms may be present in the uterus for some time. Superovulation treatments did not reactivate these infections (18), and there is little evidence that the organism has marked affinity for the zona pellucida (19). Culture studies indicate that washed (10 times) ova are brucella-free but proof based on actual transfer is lacking (20).

Suggested test methods

Serological tests identify previously infected cattle but provide little information regarding the current activity of infection. Because of widespread application of control and eradication programmes this condition could be readily controlled by specifying negative herds of origin. If this is undesirable, culture of flush fluids, wash fluids and embryos would probably provide more accurate information than serology. The use of broth cultures for embryos would allow for contact with the entire surface.

DISCUSSION

The information presented above is intended to indicate possible approaches to testing of embryos and as a starting point for further studies and discussion. It is not intended as a recommendation for adoption. Many of the published studies have been limited in scope and therefore may have failed to identify significant sources of contamination with pathogens. Numerous other studies have involved the actual soaking of embryos in fluids containing high titres of virus and thus may have overestimated the dangers of viral infection. Obviously, much remains to be done in the way of verification of test results before final conclusions and recommendations can be made. An additional general problem associated with virtually all of the test methods considered, and with all testing for certification of animals and semen, is that negative results are expected and desired. Unfortunately, negative results are virtually always subject to some scepticism. Positive results are readily acceptable as evidence of contamination but, if negative results are obtained, there are almost always some questions raised regarding test sensitivity, specificity, appropriateness of sample size, selection, or the technical competence of the testing laboratory. In the case of embryo transfer, however, there may be possible approaches to providing an extra margin of safety and thereby minimizing potential problems associated with some of these factors. The use of trypsin treatment or specific antibody exposure has already been evaluated in relation to their use to prevent transmission of disease due to the adherence of IBR virus to the surface of the embryo. If embryos are frozen, the freezing process itself may destroy some fragile agents, such as BLV, that do not even withstand a single freeze-thaw cycle very
well. Other potential methods to dislodge adherent virus could include other enzymes such as glycosidases or neuraminidase, brief exposure to low pH or mild high molecular weight detergents which would not penetrate the zona pellucida but might dislodge or even lyse virus particles. The use of substances such as alpha methyl D-mannoside that effects the release of viral glycoprotein from plant lectins may also disrupt the non-specific adherence due to affinities between glycosylated surfaces of the virus particles and the zona pellucida. It may also be useful to determine whether zona-intact embryos are highly sensitive to ultraviolet irradiation. If not, it may be possible to use this method to inactivate virus adsorbed to embryos. Obviously, many of these procedures will, for various reasons, prove to be unsuccessful but they are offered as suggestions for studies that may provide information of value in preventing disease transmission as a result of embryo transfer.

MÉTHODES DE DIAGNOSTIC PRÉSENTES ET EN COURS D'ÉVALUATION POUR RECHERCHER LES AGENTS PATHOGÈNES SUR LES EMBRYONS A ZONE PELLUCIDE INTACTE. — M.J. Van Der Maaten.

Résumé : Le transfert d'embryons peut donner lieu à la transmission d'agents pathogènes. Les caractéristiques de chaque infection détermineront le choix des méthodes de diagnostic les plus sensibles et les moins onéreuses de manière à pouvoir les utiliser en routine.

Certaines méthodes de diagnostic déjà existantes ou en cours d'évaluation, en fonction des progrès de la biotechnologie, sont réservées aux travaux expérimentaux.

Selon l'agent pathogène recherché dans l'environnement de l'embryon, le choix des prélèvements se situe à trois niveaux : 1) la vache donneuse, 2) les liquides de rinçage de l'utérus et de lavage des embryons, 3) l'embryon (représenté par les embryons dégénérés ou non transférables). Etant donné que la congélation et la décongélation des embryons peuvent inactiver un virus, il est recommandé de les analyser dans les 24 heures qui suivent leur collecte.

L'ultracentrifugation permet de concentrer un virus présent en quantités non décelables dans un milieu liquide très dilué.

L'auteur expose les méthodes permettant de rechercher les agents pathogènes de six maladies : leucose bovine, rhinotrachéite infectieuse bovine, fièvre catarrhale ovine, maladie des muqueuses, stomatite vésiculeuse et brucellose bovine.

Dans la discussion, l'auteur estime que les résultats négatifs sont souvent sujets à caution et mettent en cause la sensibilité et la spécificité de la méthode de détection, son adaptation à la taille du prélèvement, ou la compétence du personnel du laboratoire.

D'autres méthodes que la trypsinisation sont envisagées pour détacher un virus de la zone pellucide embryonnaire.

MOTS-CLÉS : Brucella abortus - Embryon - Méthodes de diagnostic - Prophylaxie sanitaire - Transplantation - Vache - Virus de la fièvre catarrhale ovine - Virus IBR/IPV - Virus de la leucose bovine - Virus de la maladie des muqueuses - Virus de la stomatite vésiculeuse.
MÉTODOS DE DIAGNÓSTICO ACTUALES Y EN PROCESO DE EVALUACIÓN PARA INVESTIGAR LOS AGENTES PATÓGENOS EN LOS EMBRIONES CON ZONA PELÚCIDA INTACTA. — M.J. Van Der Maaten.

Resumen: La transferencia de embriones puede dar lugar a la transmisión de agentes patógenos. Las características de cada infección serán determinantes para elegir los métodos de diagnóstico de mayor sensibilidad y de costo menos elevado con objeto de utilizarlos en la práctica laboratorial.

Algunos métodos de diagnóstico ya existentes o en proceso de evaluación, según los progresos de la biotecnología, sólo son utilizados en los trabajos experimentales.

En función del agente patógeno investigado en el medio ambiente del embrión, la elección de las muestras se sitúa a tres niveles: 1) la vaca donante, 2) los líquidos de enjuague del útero y de lavado de los embriones, 3) el embrión (representado por los embriones degenerados o no transferibles). Teniendo en cuenta que la congelación y descongelación de embriones pueden inactivar un virus, se recomienda que se los analice en las 24 horas que siguen a la recolección de los mismos.

El ultracentrifugado permite concentrar un virus presente en cantidades que no se pueden detectar en un medio líquido muy diluido.

El autor describe los métodos con los que se pueden investigar los agentes patógenos de seis enfermedades: leucosis bovina, rinotraqueítis infecciosa bovina, lengua azul, enfermedad de las mucosas, estomatitis vesicular y brucelosis bovina.

En la discusión, el autor estima que a menudo son discutibles los resultados negativos, poniendo en tela de juicio la sensibilidad y carácter específico del método de detección, su adaptación al tamaño de la muestra, o la competencia del personal del laboratorio.

Se contemplan otros métodos que la tripsinización para desprender un virus de la zona pelúcida embrionaria.

PALABRAS CLAVE: Brucella abortus - Control sanitario - Embrión - Métodos de diagnóstico - Transplante - Vaca - Virus de la enfermedad de las mucosas - Virus de la estomatitis vesicular - Virus IBR/IPV - Virus de la lengua azul - Virus de la leucosis bovina.

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REFERENCES


