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Moving past serology: Diagnostic options without serum

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ABSTRACT

Detecting antibodies formed in serum in response to infection is the traditional function of serology. Diagnostic modalities have included complement fixation tests, agar gel immune-diffusion, radioimmunoassay, ELISA and immunofluorescence. More recent technology now allows for the direct detection of pathogens by PCR. This review details the options for diagnostic testing using specimen types other than serum, identifying the advantages and disadvantages of these options and providing evidence for more widespread use of these techniques and specimen types.

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Introduction

A quest for clean serum specimens to detect immunological responses to the presence of important pathogens has characterised the history of disease testing in veterinary medicine. Serum has traditionally been preferred over whole blood to decrease nonspecific reactions, and give more accurate and reliable results. Assays such as the complement fixation test, dating from the very beginning of the last century (Bialynicki-Birula, 2008), agar gel immune-diffusion test (Ouchterlony, 1948), radioimmunoassay (Yalow and Berson, 1960) and immunofluorescence antibody testing (Voller, 1964) were some of the first tests used. In more recent years, ELISA (Engvall and Perlmann, 1971; Van Weemen and Schuurs, 1971) in its various permutations (direct, indirect, competitive, sandwich, capture) has been able to detect either antibody or antigen, and is popular because it is simple, inexpensive and rapid.

Newer technologies such as PCR and quantitative PCR (Mullis and Falloona, 1987) are pathogen detection methods and could readily be applied to a variety of a specimen types containing genetic material. Extraction and amplification clean-up steps now make PCR less prone to interference than earlier versions that relied on observation to detect lines or agglutination. Recent further developments in PCR technology, eliminating the need for expensive thermocyclers, have the potential to further revolutionise field diagnostics (Thekiso et al., 2007).

Specimen types other than serum have commonly been collected and tested in recent years, but difficulties of obtaining a clean and reliable signal to confirm a diagnosis have had to be overcome. Improvements in laboratory science and accessibility to good laboratory services and practices have improved diagnostic efficiency and decreased turnaround times. Additionally, efficient courier services make it practical to transport suitable specimens to a well-equipped laboratory.

In some parts of the world it is still difficult to get good quality specimens reliably to a diagnostic laboratory, either because the necessary transport infrastructure is absent or distances within the country or between countries to the laboratory infrastructure are too great. Similarly, convenience and cost-effectiveness can affect specimen collection, as obtaining a blood specimen is often the domain of veterinary or para-veterinary personnel, adding to the expense of diagnostic testing. Other readily obtainable body fluids, excretions or tissues could be obtained by less skilled personnel or animal owners to save on collection costs. In the human diagnostic field there is currently significant interest in exploring alternative specimen analysis such as dried blood spot testing for the diagnosis of hepatitis C (Coats and Dillon, 2015).

Recently, there has been a move away from centralised laboratory services, to ‘in-house’ or practice-based laboratory services for a variety of clinical disciplines, including serology. The robustness and reliability of the sampling technology has improved and has also started to venture out towards more novel applications, including point-of-care single specimen assays.

This review discusses a number of testing modalities with some examples from the authors’ experience. Since this field is constantly evolving, the examples are not comprehensive, but will hopefully encourage others to explore more novel testing options.
Milk

Milk can be a suitable medium for animal disease testing as it is generally easy to obtain (often without any specialised equipment), and in dairy cattle it is often available throughout the year. Using milk as a specimen, a wide range of animal diseases can be tested for in individual animals and in pooled specimens from herds. Tests for antibodies against the following pathogens are available: *Brucella abortus* (Nielsen and Gall, 2001), bovine viral diarrhoea virus (BVDV; Lanyon et al., 2014b), enzootic bovine leukosisis (EBL) and bovine herpes virus 1 (*BoHV1*; Reber et al., 2012), *Neospora caninum* (Schares et al., 2004; Hall et al., 2006), liver fluke (*Fasciola hepatica*; Reichel et al., 2005), Johne’s disease (*Mycobacterium avium* subspecies *paratuberculosis*; MAP; Collins et al., 2005) and *Ostertagia ostertagi* (Charlier et al., 2005; Forbes et al., 2008) in cattle. Single animal testing can be performed and tank milk from herds of dairy cows is a ready-made pool for testing groups of animals. Tank milk presents a natural pool of animal biological specimens that, with adequate test analytical sensitivity, enables the tester to screen large numbers of animals for the presence or absence of disease. While testing for EBL by antibody ELISA, milk pools rarely exceeded 100–200 cows (Ridge and Galvin, 2005), but PCR testing for BVDV is now routinely performed on pools in excess of 400 because high test analytical sensitivity provides a very cost-effective way of screening large herds and, more broadly, whole dairy industry. Herds of >400 cows are typical for the New Zealand dairy industry (Hill et al., 2010).

Historically, milk testing for pathogens was used to test for *B. abortus* infection using the ‘milk ring test’ (Fleischhauer, 1955), where a drop of stained *B. abortus* antigen was added to a pooled milk specimen. If antibody to *B. abortus* was present, an antibody–antigen complex was formed, adhering to milk fat globules and rising to the surface of the milk as a coloured ring (Fleischhauer, 1955). False positive results for *B. abortus* antibody can occur in cattle vaccinated against *B. abortus* <4 months prior to testing, or in milk containing colostrum or from cows with mastitis. Infection with other pathogens, such as *Yersinia enterocolitica*, can also cause nonspecific positive reactions (Kittelberger et al., 1997). Comparable results are obtainable with the fluorescent polarisation assay (Nielsen and Gall, 2001), and this newer test can be used in the field.

Viral pathogens such as bovine leukaemia virus (causative agent of EBL) and *BoHV1* (causative agent of infectious bovine rhinotracheitis; Witte et al., 1989) can also be tested for in milk. BVDV is widely tested for in milk specimens and testing can be simultaneously performed to detect virus (by PCR) and antibody (by ELISA), thereby providing a means of establishing both the presence and absence of the virus and measuring herd immunity. Antibodies formed against BVDV are excreted into milk and correlate well with serum antibody titres. Pooled testing provides a quantitative ELISA assessment strongly correlated with within-herd prevalence (Lanyon et al., 2014b). High levels of antibody in the tank milk suggest sufficient exposure to virus to reduce the need for vaccination, while low tank milk antibody levels suggest the need for biosecurity measures or vaccination to prevent infection. The ability to monitor the relative changes in pooled antibody levels, and therefore, within-herd prevalence is particularly valuable. A sudden increase may be indicative of a recent infection of infection, a change that can be difficult and expensive to detect when relying on individual animal testing, and would suggest the need for further investigation, beginning with bulk milk PCR testing.

As a logical follow-on after eradication of BVDV from the national dairy herd in Switzerland, on-going monitoring for continued freedom from BVDV infection is now based on regular bulk milk surveillance in that country (Presi et al., 2011). Surveillance testing for recently emerging animal diseases, such as bluetongue and Schmallenberg viruses, has also been based on bulk milk testing by ELISA (Balmer et al., 2014).

Pooled milk testing has also been successfully applied for EBL detection. Screening of all dairy herds in New Zealand by testing pools of milk from groups of 20 dairy cows using EBL ELISA found no evidence of infection by 2011 (Voges, 2011). While primary testing was on pooled milk specimens, further testing was required for any suspicious or positive results using individual serum antibody ELISA and PCR tests. By contrast, the EBL status of the beef industry in New Zealand remains unknown.

In Switzerland, a bulk tank milk specimen to test for *BoHV1* reported significant cost savings by using milk instead of serum to detect antibodies. If the expenditure was identical, testing bulk milk yielded significant increases in test sensitivity (Reber et al., 2012), thus improving diagnostic outcomes.

Diagnostic outcomes have also been improved through the use of *N. caninum* bulk milk ELISA testing to predict the prevalence of infection in dairy herds in Australia (Hall et al., 2006). The stage of lactation affected the accuracy of the comparison between serum and milk (Schares et al., 2004), and milk testing was more sensitive. Excellent accuracy has also been demonstrated when testing individual milk specimens for antibodies against *F. hepatica*, with very high sensitivity and specificity close to 100%. However, when bulk tank milk specimens were tested there was a decrease in sensitivity, so only dairy herds where the prevalence of *F. hepatica* was >60% could be identified (Reichel et al., 2005).

Serological tests for Johne’s disease have low sensitivity but reasonable specificity. Testing of individual milk specimens yielded a sensitivity of 28% (Collins et al., 2005), slightly higher than serum, and sensitivity increased with age of animal tested (Nielsen et al., 2013). PCR can also be used to test for the presence of MAP DNA in milk (Buergelt and Williams, 2004), as can the peptide-mediated magnetic separation-phage (PMS-phage) assay (Foddai et al., 2011). However, advances in PCR testing for MAP in faeces could negate the need to use antibody based tests.

Antibody based tests (ELISA) are available to measure antibodies in bulk tank milk to the abomasal parasite *O. ostertagi* (Forbes et al., 2008). Only an association between ELISA values and milk yield can be made using these test results, rather than confirming true positive nematode infections in the herds, so additional diagnostic testing is required to establish the parasite status of the herd.

In sheep, Q-fever (*Coxiella burnetii*; Klaasen et al., 2014), *Brucella melitensis* (Hamidi et al., 2015) and *Mycoplasma agalactiae* (Poumarat et al., 2012) can be tested for using milk; in goats, milk specimens can be used to test for caprine arthritis and encephalitis (*Nagle-Alne et al., 2015*). Q-fever outbreaks in humans are associated with *C. burnetii* infection in small milk inguminants in Africa (Klaasen et al., 2014). Shedding of the organism is intermittent, thus infection was not always detected by PCR and serological tests might also be required. In contrast, PCR testing of milk for *B. melitensis* detected was more sensitive than serology in one study (Hamidi et al., 2015). Accurate serological classification of the *M. agalactiae* status of sheep is difficult and PCR testing of milk specimens with two PCRs should be used to confirm the presence of the organism. The resultant PCR results also require cross checking with a dot-immunobinding technique (Poumarat et al., 1991).

Milk testing can be utilised for detection of non-infectious conditions. For example, lateral flow devices to test for progesterone concentrations in milk present opportunities to define the oestrus cycle and pregnancy status of cows (Waldmann and Raud, 2016) and technological modifications may allow for testing to occur during milking (Dobson, 2016).

Colostrum

Colostrum is another medium that can be used for animal disease testing instead of milk. Its availability is restricted to a shorter time period, but provides other testing and diagnostic advantages. Testing
colostrum for antibodies (as an alternative or add-on to milk testing) is potentially useful because of the higher concentration of immunoglobulins in colostrum compared to milk. The concentration of IgG is estimated to be up to 100 times higher than milk in the first few days after parturition (Korhonen et al., 2000). Recent work in cattle suggests that testing colostrum increases analytical and diagnostic sensitivity compared to milk. This could be most useful for on-going surveillance of animal diseases requiring less frequent checks, as colostrum is only available during the perinatal period (Jenvey et al., 2012, 2015; Cockcroft et al., 2014).

The drawback of colostrum testing is the small window of availability and the difficulty of collection in some species. In dairy cattle, sampling of colostrum might be most usefully applied for diseases where diagnostic tests are hampered by low analytical and/or diagnostic sensitivity (such as the case with Johne’s disease; Reichel et al., 1999). Colostrum has also been used successfully for testing for rotavirus and mycoplasma infections (Corthier and Franz, 1981; Zimmermann et al., 1986; Rautiainen, 1998).

Hair and ear notch skin specimens

Hair specimens can be easily obtained, even by animal owners, and submitted for testing. Meanwhile, ear notches may be the by-product of routine farming procedures such as ear-tagging, thereby reducing the amount of additional handling and restraint required for animal disease testing.

Hair and ear notch specimens have been used successfully to detect BVDV persistently infected animals (Hill et al., 2007; Lanyon et al., 2014c) and formed the basis of the recent successful Swiss BVDV eradication campaign (Presi and Heim ,2010). In this campaign, detection of BVDV antigen in skin was the choice of choice to identify persistently infected calves, and was preferred over serum. After the ingestion of colostrum, maternal anti-BVDV antibodies can bind to BVDV antigen and prevent its successful detection in the routinely used antigen-capture ELISA (Fux and Wolf, 2012). Using ear notch skin specimens reduces this complication, as there are fewer antibodies in ear notch tissue. Heating of serum specimens under specific conditions to break up antibody-antigen complexes can overcome the interference of maternal antibody and allow successful serum testing. This adds extra steps to the procedure, but has been used effectively in BVDV testing (Lanyon and Reichel, 2016) and in heartworm serology (Little et al., 2014a, 2014b; Velasquez et al., 2014).

Ear notch specimens are increasingly being used for parentage testing because of the ease with which they can be obtained¹. Hair has also been used (Singh et al., 2011) to identify persistently BVDV infected animals, and in parentage testing² and DNA profiling.

Swabs from mucosal surfaces

Swabbing of readily accessible animal surfaces is an option for diagnostic testing in animals that could otherwise be difficult to restrain for more invasive blood sampling. Swabs from mucosal surfaces, including the mucous membranes of the eyes, vagina, prepuce, and oral cavity can yield antibody and antigen in sufficient quantities for testing to produce meaningful results (Lanyon et al., 2014c). Swabs can also be used for parentage (DNA) testing and have been used to detect the following pathogens: Bordetella bronchiseptica, Leishmania, feline leukemia virus (FeLV), feline herpes virus 1 (FHV-1), Chlamydia felis, feline calcivirus (FCV), BoHV1, bat lyssavirus, Mycoplasma felis and rinderpest virus (Mushii and Wafula, 1984; Lutz and Jarrett, 1987; Echevarria et al., 2001; Strauss-Ayali et al., 2004; Di Muccio et al., 2012; Hernandez et al., 2015; Litster et al., 2015).

The characteristics of specific disease states can influence the usefulness of these tests. One study found that 96% of latently infected cats did not shed FeLV RNA in saliva and were not detected by PCR (Gomes-Keller et al., 2006a), but virus shedding was a consistent feature in viraeic cats (Gomes-Keller et al., 2006b). A conjunctival swab tested by PCR had the highest sensitivity compared to immunofluorescent antibody and lymph node microscopy for the diagnosis of Leishmania, but still only detected 74% of infected dogs (Di Muccio et al., 2012). Screening bats for lyssavirus with PCR using oropharyngeal swabs was more accurate than PCR of fresh brain (Echevarria et al., 2001), and could be performed antemortem. Combination PCR testing of oropharyngeal swabs and either conjunctival or nasal swabs were needed to identify all cats infected with B. bronchiseptica, FHV-1, C. felis, FCV, and M. felis in one study (Litster et al., 2015), but a single PCR test could be performed on both specimens. One-step multiplex PCR testing of nasal swabs and bronchoalveolar lavage specimens from cattle for BoHV1, bovine respiratory syncytial virus and bovine parainfluenza virus 3 was more sensitive than virus isolation and had a sensitivity of 97% in another study (Thonour et al., 2012).

Preputial and vaginal swabs can be tested in cattle for the presence of Campylobacter fetus spp. venerealis (McMillen et al., 2006) using a 5’ Taq PCR assay. Subsequent studies found there was cross reaction between C. fetus spp. venerealis and Campylobacter hyointestinalis (Spence et al., 2011), illustrating the need for additional confirmatory testing of any PCR positive C. fetus spp. venerealis isolates. Improved sensitivity in detecting Trichichomonas fetus in smegma and vaginal mucous was demonstrated using PCR (McMillen and Lew, 2006) compared to culture. Quantitative PCR testing for Ureaplasma diversum was 100-fold more sensitive than conventional PCR (Marques et al., 2013). In addition, buccal swabs can also be collected to test for a range of inherited diseases including collie eye anomaly (Chang et al., 2010), hereditary cataract (Mellersh et al., 2006), and ceroid lipofuscinosis in dogs (Karli et al., 2014). Collie eye anomaly can be diagnosed by examination of the retinas in 5–10 week old puppies, but after 12 weeks this is impossible as the lesions become covered with pigment. Saliva collected onto filter paper from Collie-related breeds of dog of any age can rapidly be tested for the anomaly with the cost effective SYBR green method (Chang et al., 2010) allowing for large scale screening of populations. Saliva can also be collected for real time PCR testing for ceroid lipofuscinosis in dogs (Mizukami et al., 2011), providing early opportunities for diagnosis rather than waiting for the onset of clinical signs and confirmation by histopathology.

Urine

Urine specimens are relatively difficult to obtain in animals, requiring direct capture of voided specimens with risks of contamination. Alternatively, urinary catheterisation or cystocentesis of individual animals is needed if a clearly identifiable uncontaminated specimen is required. Urine has been used as a medium for testing animal diseases and is particularly useful to test for Leptospira infection. Increasingly, PCR is used as the method of choice for the detection of the organism (Chappel et al., 1985; Fang et al., 2014; Hamond et al., 2014). Urine testing is also used to detect if performance enhancing drugs have been used in horses (and humans athletes; Thevis et al., 2016).

¹ See: http://www.genomnz.co.nz/ear-punch-sampling-new-method-for-genomnz/ (accessed 18 April 2016).
² See: http://www.genomnz.co.nz/how-to-take-dna-samples/hair-samples/ (accessed 18 April 2016).
Semen

Semen of male animals is frequently tested to screen for infection, mostly reproductive pathogens such as Brucella ovis in rams, N. caninum, BoHV1 and T. fetus in cattle, but also viruses such as equine infectious arteritis (Campero et al., 1990; Ramina et al., 1999; Ortega-Mora et al., 2003; Serrano-Martinez et al., 2007; Wang et al., 2007; Rana et al., 2011; Ridler et al., 2014). Specimens can be difficult to obtain in pastured animals and may require sedation, specialised equipment and collection procedures. However, diagnostic testing of semen is particularly valuable for ensuring semen collected from bulls at semen collection centres is free from C. fetus spp. venerealis (Eaglesome et al., 1995), BVDV (Rikula et al., 2008) and BoHV1 (Alegre et al., 2001) to ensure venereal infection is not transmitted via natural or artificial breeding.

Faeces

Testing for the presence of pathogens in faeces is a preferred option for diseases that are spread via excretion in the faeces, and has certain advantages over serological testing. For example, for E. histolytica, serological tests perform very well, demonstrating high diagnostic sensitivity and specificity (Reichel, 2002) and the detection of antibodies confirms previous exposure to the parasite with high accuracy. However, antibodies may persist for weeks after an infection has been eliminated by effective treatment with a flukicide. In order to detect patent infections (with shedding), faecal (copro-)antigen ELISAs have successfully been deployed to detect active, patent infections with a high degree of diagnostic sensitivity (and perfect specificity; Palmer et al., 2014), thus giving an indication of current infection. Similarly, the application of diagnostic assays to detect the presence of nematode larvae and eggs in faeces presents new options for investigation of the parasite infection status of sheep and cattle, using a combined microscopy-molecular technique where parasite eggs are separated from faeces by a salt flotation technique before PCR testing to identify the species present (Bott et al., 2009; Hoglund et al., 2013). This avoids the need for time-consuming larval culture and identification.

Testing for antibodies to Johne’s disease is hampered by the late onset immunological (particularly humoral) response, limiting the diagnostic sensitivity of serological tests (Reichel et al., 1999). Culture is similar to serological testing in its ability to detect infected animals, although it was considered the reference standard for testing. Faecal culture enhanced by PCR testing has improved diagnostic sensitivity and is now the new reference standard (Whittington et al., 2000; Marsh and Whittington, 2001).

Faecal ELISA testing can be used to detect rotavirus in foals, (Browning et al., 1991), Giardia duodenalis in cats (Mirecan et al., 2011) and Cryptosporidium parvum (Anusz et al., 1990), Escherichia coli K99 and coronavirus in cattle (Cho et al., 2012). Faecal PCR testing enables identification of enterotoxigenic E. coli from diarrhoeic piglets (Byun et al., 2012) and T. fetus infection in cats (Gookin et al., 2002).

Point of care test devices

Very recently, the diagnostic test devices themselves have been shown to be suitable as specimens, as foot-and-mouth disease virus was recovered successfully from lateral flow devices (Fowler et al., 2014), further expanding the possible range of sampling modalities.

The concept of specimen pooling

Pooling of specimens can be used to provide information about immunological reactions to specific pathogens, or to identify pathogens or reagents of interest. Often this method is used for economic reasons; testing a large number of pooled specimens with one test is generally far cheaper than testing each animal individually. This is the case where the additional individual diagnostic test adds considerable cost, but the specimen is relatively cheap and readily available (e.g. milk). Pooled specimen testing is particularly useful as a screening test for the absence of disease or where disease prevalence is low. While pooling offers a method of investigating the disease status of a large population quickly (Lanyon et al., 2014a), there are two points important to note. Firstly, when testing for the presence of a condition/factor or pathogen, pooling only makes sense if it makes testing more cost-effective or less time-consuming than individual testing. If the prevalence of the factor of interest is higher than the dilution factor caters for, all pools will be positive, and nothing has been gained. For example, if you pool 20 animals/pool for BVDV virus testing and the disease prevalence in the population is 1–2%, 80% of pools are likely to screen negative, saving time and money. However, if you test 100 specimens/pool from the same population, all pools are likely to be positive, and you are no closer to identifying which animals are infected. By contrast, a run of 100 specimens is within the analytical capability of a PCR assay. Secondly, the diagnostic test also needs to possess sufficient analytical sensitivity to detect the antibody or antigen at the dilution factor determined by the degree of pooling. Usually an additional safety factor is added to ensure this. When testing for antibodies to EBL, the greatest dilution allowed for pooling is 10 times less than the highest dilution of a standard positive that can be detected e.g. if the standard can be detected diluted 1:100, pools of 10 animals each can be tested (Reichel et al., 1998).

One of the issues with pooling is the loss of the ability to account for the influences of individuals with very strong relative contributions, such as an animal with a very high antibody titre, distorting the overall picture. Hence, pooling generally indicates the level of infection or immunity at the herd, flock or unit level. For example, when testing for BVDV antibodies by ELISA, a single strong positive individual can result in a positive result in a pool of up to 128 individuals, while even a weak positive individual can cause a positive result in a pool of eight (Lanyon et al., 2014a). On the other hand, pooling provides a very cost-effective snapshot of that herd, flock or unit and can, if interpreted appropriately and used in conjunction with antibody and pathogen testing, can provide a very effective ongoing surveillance tool to augment biosecurity.

Conclusions

In addition to serum, a variety of animal excretions, secretions and tissues can successfully be used for the detection of an immune response to an agent, or for detection of the agent itself. Improving detection technologies present exciting future options for disease diagnosis and control. Drivers for exploring different test media have, in some cases, been the quest for more cost-effective testing modalities or an effort to make use of specimens that are more readily available and can easily obtained by the farmers themselves.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with people or organisations that could inappropriately influence or bias the content of the paper.

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