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White Paper

Multiplex Methods in Veterinary Medicine and Agriculture

Introduction

The fields of veterinary medicine and agricultural science encompass an array of subspecialties, including livestock and crop production, food and water safety, translational research, companion animal medicine, zoonotic disease, wildlife and ecosystem health, and more.[1] This broad landscape presents virtually unlimited research challenges, many of which require accurate measurement of biochemical processes.

Over the past decade, such needs have been increasingly met by systems using xMAP® Technology. Using color-coded beads known as microspheres, xMAP-based multiplex assays can detect up to 500 analytes in a single, small volume sample, making them more time and cost efficient than traditional methods.[2] While Luminex and its partners offer one of the most extensive commercially available assay menus that use xMAP Technology, customers also have the option to design custom assays for novel research.

Potential areas of application for xMAP Technology are as far-reaching as the fields of veterinary medicine and agricultural science. For instance, in veterinary research, xMAP Technology may be used to study host immune proteins and infectious agents. Further, with significantly smaller sample volume requirements than traditional immunoassays, xMAP Technology makes it possible to study birds and other small animal species. In the agricultural field, xMAP-based assays may be used for food safety, as they enable the detection of foodborne pathogens, hormones, antibiotics, and other drugs. Beyond testing specimens from animals, multiplex assays can also be used to measure various mycotoxins in feed, differentiate viruses in plants, and evaluate the quality of water and other environmental substrates.

This article offers a review of such applications in recent veterinary and agricultural research. As the studies demonstrate, xMAP Technology can meet a wide variety of diagnostic needs with both existing kits and custom assays.

Existing Kits

Comparative medicine applications

Using xMAP Technology, investigators can characterize immune responses in candidate animal models, thereby evaluating the suitability of animal species for studying human disease, as demonstrated by the following two studies.

Gardner et al. employed a MILLIPLEX® MAP Porcine Cytokine and Chemokine Magnetic Bead Panel to evaluate the local and remote effects of acute kidney injury (AKI) in pigs.[3] Using the array, the investigators measured 11 cytokines in the liver and lungs after ischemia-reperfusion AKI. The panel showed that none of the cytokines were significantly altered in either location. This, coupled with other findings, suggested a lack of remote organ damage. These findings differ from studies of human AKI, in which immune-mediated damage has been observed in remote organs. According to the investigators, this difference between species—uncovered by the xMAP-based array—may discourage the use of pigs as a large-animal model for remote organ injury in human AKI.

In response to the outbreak of H7N9 avian influenza virus in China, Shichinohe et al. conducted research with cynomolgus macaques, a candidate model for human infection.[4] After inoculating macaques with four types of H7 avian influenza virus (H7N1, H7N2, H7N7, and H7N9), the investigators characterized immune responses by testing serum and nasal swab samples with the **MILLIPLEX® MAP Non-Human Primate Cytokine Magnetic Bead Panel**. The assay, which can detect up to 23 pre-mixed analytes, was used to measure MCP-1, MIP-1α, MIP-1β, TNF-α, IL-1β, IL-6, and IL-8, with cytokine results characterizing the pathogenicity of each viral subtype. The comparison showed that H7N7 and H7N9 were more pathogenic than H7N1 and H7N3. These findings differed from previous studies involving chickens and aligned more with human research, suggesting that macaques may be more suitable than chickens for gauging pathogenicity of H7 avian influenza viruses in humans.

Companion animal medicine

For a similar study of viral immune responses in cats, Safi et al. turned to another existing kit, the**MILLIPLEX® MAP Feline Cytokine/ Chemokine Magnetic Bead Panel Premixed 19 Plex**.[5] With this assay, the investigators measured the expression of 19 immune-related proteins in samples from cats infected with feline coronavirus, and in culture, infected Crandell-Reese feline kidney cells. The panel showed that IL-8, KC, RANTES, and MCP-1 were expressed in culture. In serum from infected animals, and, when present, peritoneal effusion, all 19 immune-related proteins were detected. While cytokine profiles did not differ between cats with or without peritoneal effusion, the level of expression was higher when effusion was present, suggesting that a clinically relevant diagnostic could be possible.

Johnson et al. employed a similar xMAP-based assay to study sepsis and immune-mediated hemolytic anemia (IMHA) in dogs.[6] Using a MILLIPLEX MAP Canine Cytokine/Chemokine Magnetic Bead Panel, the investigators measured 14 cytokines in dogs that were healthy or in the early stages of sepsis or IMHA. After testing samples from healthy dogs to establish cytokine reference ranges, the investigators grouped cytokines into four categories based on function, and then compared changes in these groups between dogs with sepsis and those with IMHA. Results showed that both groups had similar cytokine profiles, indicating a shared immune pathway that was independent of disease etiology. According to the authors, this finding could lead to a shared immunotherapeutic intervention for distinct immune-mediated diseases.

Nutrition research

To determine the impact of dietary protein levels on the body composition of dogs, Kawauchi et al. used the **MILLIPLEX® MAP Canine Gut Hormone Magnetic Bead Panel**.[7] The investigators looked at insulin, leptin, and glucagon, and among 14 dogs fed two different levels of protein, these hormones were measured in serum before and after neutering, at weeks 4, 12, and 26. Only glucagon was significantly associated with protein levels. Although glucagon level was significantly higher among dogs that received more protein, the magnitude of this increase was still within the reference range, suggesting that the finding had little or no clinical relevance.

Food safety applications

Xie et al. used the **xMAP *Salmonella* Serotyping Assay** to characterize environmental *Salmonella* enterica of three beef cattle feedlots in Texas.[8] First, the investigators collected multiple sample types: feed from feed bunks, dropped feces, drinking water from troughs, and soil in cattle pens. After pooling by feedlot and type, presumptive *Salmonella* isolates were analyzed using the xMAP assay, revealing six serovars: Montevideo, Kentucky, Kralingen, Muenchen, Altona, and Anatum, with the latter being the most common. Culture of *Salmonella bacteriophages* showed that the one feedlot without *Salmonella* maintained a reservoir of phages, suggesting that phages may inhibit the survival of *Salmonella* in feedlots.

Pharmacological research

In another study, DeOliveira et al. used the **MILLIPLEX® MAP Mouse Adipokine Magnetic Bead Panel** to determine the effects of three compounds on obesity in mice.[9] By measuring 5 of 7 available analytes (TNF-α, IL-10, leptin, adiponectin, MCP-1, glycerol), the investigators found that one agent, an A_{2A} receptor agonist, reduced inflammation in serum and visceral adipose tissue, suggesting that the compound could lead to the development of novel anti-obesity therapies. This study highlighted the use of small-volume biological samples, for which xMAP-based assays are ideal.

Wildlife and exotic animal medicine

While custom xMAP-based assays can be designed to meet unique research needs, there are kits available for domestic animals that may be used with exotic species. For example, Levin et al. validated the cross-reactivity of the **MILLIPLEX® MAP Canine Cytokine/Chemokine Magnetic Bead Panel** in three pinniped (seal) species.[10] In seals, the canine multiplex assay was able to detect TNF-α, INF-γ, IL-6, IL-8, and IL-10, potentially aiding in the diagnosis of subclinical infections and immune responses.

Custom Kits

If an xMAP-based kit isn't available for a particular area of research, a custom multiplex assay may be developed.

Comparative medicine

In 2018, Hussain et al. used a custom xMAP-based multiplex assay to study canine mammary tumors, which may serve as a model for human breast cancer.[11] The panel was designed to detect autoantibodies for 5 tumor-associated autoantigens: PGAM1, TPI, MNSOD, MUC1, and CMYC. Using this custom assay, the investigators tested the serum of 125 dogs—75 of which had mammary tumors, and 50 of which were healthy. Results showed that positivity of the combined panel was associated with the presence of mammary tumors, based on an area under the curve (AUC) of 0.931. In the clinic, this assay could test dogs for mammary tumors in less than three hours with just one microliter of serum.

Equine medicine

In a more acute setting, Fogle et al. developed a serum-based assay to identify horses with endotoxemia.[12] Using xMAP Technology, the investigators measured soluble CD14, an early inflammatory marker, in 19 clinically endotoxemic and 36 clinically nonendotoxemic horses. The median CD14 level was significantly higher in the endotoxemic group than the nonendotoxemic group (1,102 vs. 692 ng/mL), suggesting that CD14 may be useful for early identification of horses with endotoxemia.

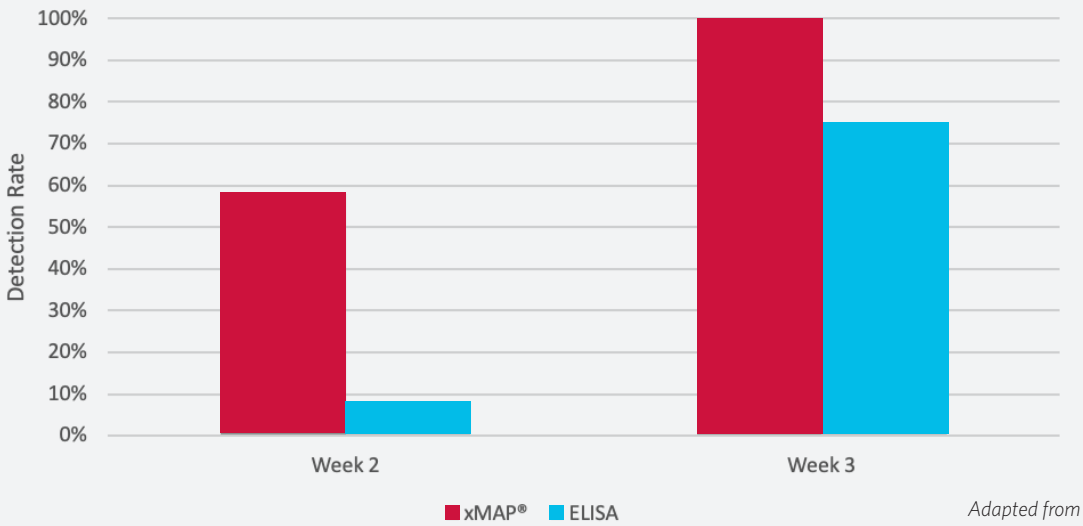
Animal model diagnostics

To create a novel, serum-based diagnostic test in rats, Xu et al. employed **MagPlex-TAG™ Microspheres**, which can detect up to 150 different nucleotide sequences in a single reaction.[13] The investigators selected sequences for four rat parvoviruses, all of which may cause subclinical infections in laboratory populations, and can be challenging to detect with existing serological assays due to cross-reactivity. Compared with conventional PCR, the xTAG®-multiplex PCR assay detected the viruses in tissue samples more frequently, and subsequent testing showed that this panel was suitable for fecal samples as well.

Food production

Similarly to Xu et al., Gerber et al. used xMAP-based technology to detect subclinical infections—this time in chickens.[14] The investigators developed an assay that would detect anti-avian hepatitis E virus (HEV) immunoglobulin Y antibodies. This panel and a conventional ELISA were used to test 96 chickens with known exposure to HEV, and revealed that the two tests demonstrated high agreement (κ = 0.63). However, a subsequent experiment involving 12 infected chickens showed that the xMAP-based assay could detect infection significantly earlier than ELISA. At two weeks, the xMAP-based assay detected infection in 7 out of 12 chickens (58%), compared with 1 out of 12 (8%) for the ELISA. At three weeks, the xMAP assay detected 12 out of 12 infections (100%), compared with 9 out of 12 (75%) for the conventional method (**Figure 1**).

Figure 1. Hepatitis E Virus Infection Detection Rates



Adapted from Gerber et al.[14]

Food safety applications

Using a novel xTAG assay to enhance Salmonella serotyping, Zheng et al. amplified the O, H, and Vi antigen genes from 228 Salmonella isolates. Then, they identified the PCR products of these different antigens using MagPlex-TAG microsphere hybridization. This technique had a sensitivity and specificity of 95.1% and 100%, respectively, compared with conventional serum agglutination testing. The investigators concluded that their custom xTAG assay enhanced Salmonella serotyping in multiple ways: the new assay could be used with both MAGPIX® and Luminex® 200™ systems, it needed only primers instead of primers and probes, and coupling of antigen-specific oligonucleotide probes to beads was unnecessary.

Zoonosis and biodefense research

Brucella, a bacterium that is highly infectious through aerosols, has been identified as a potential bioterrorism agent, necessitating tests that are capable of rapid, highly sensitive detection. To develop such an assay, Silbereisen et al. leveraged xMAP-based technology. By detecting monoclonal antibodies for the lipopolysaccharide (LPS) O-antigen of *Brucella* species, the assay could identify *Brucella* with detection limits ranging from 2×10^2 to 8×10^4 cells/mL, depending on the species. The assay was capable of detecting *Brucella* in milk, and integration into a multiplex panel allowed for single-sample testing for *Brucella* species, *Francisella tularensis, Yersinia pestis,* and *Bacillus anthracis.*[16]

Commercial crop production

While the previous examples have focused on animals, xMAP Technology can also be used with plants.

Bald-Blume et al. used 11 types of MagPlex-TAG Microspheres to detect plant-infecting tospoviruses.[17] Along with general detection, this assay could simultaneously differentiate viral species, including Impatiens necrotic spot virus, Tomato spotted wilt virus, Watermelon silver mottle virus, and Capsicum chlorosis virus. This was an important step forward in the field, since commercial antibodies and conventional ELISA face issues with cross-reactivity. Further, the xTAG method allowed for multiplexing—overcoming the need for multiple independent tests, which are necessary when differentiating species via conventional ELISA.

A second plant study by Zhang et al. used xMAP-based methods to detect 8 fungicide-resistant alleles in the causal agent of gray mold, *Botrytis cinerea*.[18] Simple and high-throughput detection of fungicide resistance is needed for gray mold, which can affect a wide range of vegetables, fruits, bulbs, and ornamental plants. The xTAG assay developed by Zhang et al. showed no cross-reactivity between probes for sequence variants. Depending on the allele, the assay could detect between 0.45% and 4.5% of mutations mixed within the genomic DNA background, suggesting high sensitivity.

Summary

The studies described here, including viral diagnostics in companion animal species, environmental testing in livestock feedlots, and fungal resistance detection in commercial crop production, show that xMAP-based assays are widely adaptable, and can support research and diagnostics across multiple fields, including veterinary medicine and agricultural science.

The studies also reveal how xMAP-based assays can bridge the gap between veterinary medicine and human health. Using xMAP Technology, investigators are determining appropriate animal models for human disease, while others are developing scalable diagnostics that are more efficient and cost-effective than conventional methods. This latter need is particularly significant, as 75% of emerging infectious diseases in humans come from animal species.[19] It is therefore essential to develop novel diagnostics that can rapidly and accurately detect pathogens on a large scale in both animal and human populations.

Finally, in commercial crop production, high-throughput xMAP Technology has opened new doors in plant virus research.[20] Still, much work lies ahead with regard to biological characterization of plant viruses, including how these viruses may affect biosecurity, regulation, and commerce.

Ultimately, the scale and diversity of the challenges facing investigators in veterinary medicine and agriculture ensure that xMAP-based multiplex assays will be essential in the years to come.

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