Why diagnostics are an essential part of strategic mycoplasma control

Diagnostics and monitoring are an essential part of any disease control strategy in veterinary medicine. Without the knowledge on how certain interventions impact on disease development in a flock it will be impossible to devise an effective monitoring strategy.

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This is also true in mycoplasma control. With mycoplasma it might even be more important, as mycoplasma disease seldom comes unaccompanied by other diseases.

In certain areas of the world, Mycoplasma gallisepticum (MG) and Mycoplasma synoviae (MS) exhibit high prevalence rates within intensive poultry production. This is significant because they possess the ability to interfere with the immune system and aggravate the damage caused by secondary pathogens.

In addition to respiratory and joint problems, MG and MS cause great production losses, both in laying and breeding hens and in broilers. They may affect fertility, embryo viability, egg production and quality in breeding and commercial hens. All of this will be further complicated by common secondary and superinfections.

Without information on actual disease presentation in the field and its influence on production data, the results of diagnostic tests such as ELISA or PCR lose most of their meaning.

A more holistic approach is called for: this needs to address diagnostics and timely interventions such as treatment, vaccination and biosecurity. In this article the choice and usefulness of diagnostic methods for mycoplasma control in poultry will be discussed.

Introduction to diagnostic (analytical) methods

For laboratory analysis, classical methods for direct and indirect detection such as serology and PCR come to mind instantly. However, equally as important are resistance testing methods such as minimal inhibitory concentration testing (MIC). Also, modern techniques based on molecular methods are emerging, such as whole genome sequencing that has been previously hampered by its almost prohibitive costs.

Isolation is needed for resistance testing

Within the group of direct tests, the gold standard for mycoplasma diagnosis is isolation.

Isolation is also the prerequisite for doing resistance testing, for example via the minimal inhibitory concentration test (MIC). Currently, this is the method of choice to evaluate the resistance profile of mycoplasma strains. MIC has a high throughput and can be easily standardised.

MG and MS are very fastidious organisms that need a specific culture medium to grow. Frey medium is usually used, and it can take up to 10 days or more to obtain a positive result. This is revealed by a colour change in the broth and the typical 'fried egg' shaped colonies on the agar. Confirmation of isolation should be made by PCR or immunofluorescence.

Obtaining MIC values becomes increasingly important to establish the antibiotic resistance profile of local strains and choose the correct course of treatment.

MIC data is available all over the world. However, it is especially crucial to have MIC for each operation so the correct control strategies for each region can be determined. Information on MIC levels is not available in many poultry operations, production levels or even on a national level.

Due to these difficulties in isolation, there is ongoing research into new techniques to determine the resistance of mycoplasmas to antibiotics. One example is the detection of mutations in the 23s rRNA gene, suspected to decrease the susceptibility of mycoplasma to macrolides. Investigations are ongoing to determine if those mutations may be used as a molecular marker to set breakpoints. Proteomics – the study of proteins produced by an organism – can be used to detect how mycoplasma is able to produce or modify its enzymatic protein expression. Theoretically, enzyme activities can then be taken as an indicator of development of resistance to different antibiotics.

PCR and other molecular tools

Molecular detection takes an ever increasing importance in mycoplasma diagnostics. The most popular direct detection technique is named after its English abbreviation, Polymerase Chain Reaction (PCR). It has excellent sensitivity and specificity. Unlike isolation, it cannot differentiate between live and dead organisms.

Samples for PCR can be taken either through choanal or tracheal swabs, organ macerations or joint fluid. DNA is extracted and can be submitted to either employ PCR methods such as real-time PCR or classical PCR with further sequencing. PCR can detect MS and MG from the same sample at the same time. Semi-quantitative PCR (qPCR) is especially useful to determine the amount of genetic material.

The amount of genetic material in a sample is quantified by determining how many cycles it takes to make that material visible. The unit used to measure the required cycles is called the cycle threshold. qPCR can be used to determine the effect of interventions on the mycoplasma load in the field.

PCR, followed by sequencing on the other hand, is a prerequisite for establishing phylogenetic trees that allow conclusions on epidemiology.

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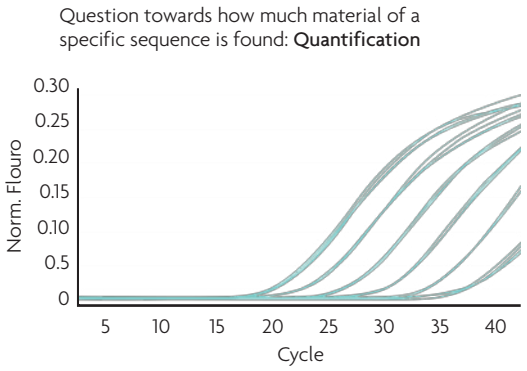
MS	Day X		MS	Day X+10	
	Sample	CT Value		Sample	CT Value
Flock 1	qPCR1	22.85	Flock 1	qPCR1	31.14
	qPCR2	22.21		qPCR2	Neg
	qPCR3	22.23		qPCR3	30.13
	qPCR4	22.00		qPCR4	26.22
Flock 2	qPCR1	21.85	Flock 2	qPCR1	Neg
	qPCR2	23.01		qPCR2	27.99
	qPCR3	23.42		qPCR3	27.00
	qPCR4	22.90		qPCR4	28.22



Cycle threshold (CT value): The more material is copied, the fewer cycles are needed to show the result on the screen. LOW CT value = a lot of DNA template to start with. HIGH CT value = little DNA template to start with.

Fig. 1. The usefulness of semi-quantitative PCR to examine treatment success.

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Epidemiology is employed to differentiate between field and vaccine strains but also allows multiple conclusions on the success of strategic control. Fig. 1. illustrates the usefulness of semi-quantitative PCR to examine treatment success.

Treatment has the capacity to reduce the overall mycoplasma load as demonstrated by an increase of the cycle threshold. Interpretation of results needs to be handled with care as qPCR also has the capability to detect non-viable mycoplasma.

This has to be taken into account when determining the sampling timepoint in such circumstances.

With the intermittent shedding patterns of mycoplasma, PCR is an especially useful tool to investigate current infection status and to help interpret serology results.

PCR is used frequently to determine if birds are already infected at certain timepoints. A good example is the timepoint of transfer of pullets to the laying house, where PCR results might indicate a mycoplasma presence that has not triggered the development of sufficient antibody levels yet. If mycoplasma is present in the flock, those antibody levels are prone to rise after the stressful transfer event or the onset of lay.

Serology – the basics

Serology is an indirect technique to detect the reaction of the animal to a pathogen through the measurement of antibody levels. The validity of these methods depends largely on correct sample planning and execution. The interpretation of the antibody levels of a flock is only significant when the sampling is reliable and representative, and the sample has been stored and treated appropriately. The coefficient of variation (%CV) is an important measure of the validity of a serological test as it is the expression of the standard deviation needed to interpret the results on a statistically sound basis. The sample size for reliable and reproducible results is of crucial importance. Numerous publications have explained how to arrive at a proper sample size.

As it is common in the field that serology sample sizes are often inadequate, it has to be made clear that too few samples significantly decrease the reliability of average titres and vaccination estimates.

The correct time point of sampling is also of paramount importance. Serum samples are best taken on a previously agreed time frequency or at least in pairs to determine if titres are rising or failing.This will allow important conclusions on disease evolution and depending on field pressure, also on intervention success. This information is so crucial that ELISA in particular, is almost always recommended for breeder but also layer flocks.

ELISA

The ELISA technique is based on the detection of an antigen or antibody by binding it with its opposite on a 96 well plate. In all serological methods a degree of cross-reactivity can be observed depending on the chosen binding agents and their immunological properties. The same is true for ELISA.

It has the great advantage of being relatively inexpensive, reasonably quick, satisfyingly specific and sensitive, as well as easily automatable. It allows for a quantitative interpretation of titre ranges and disease evolution and thus is of prime importance in a slow spreading disease such as mycoplasma.

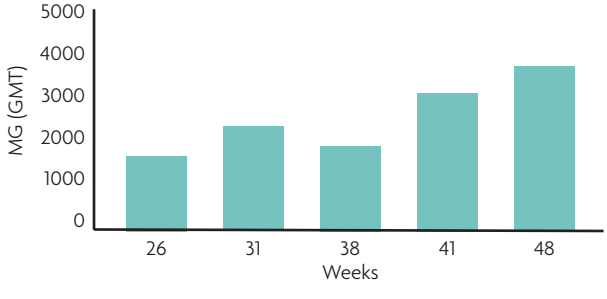
The interpretation of the results requires some experience, usually obtained by analysing and comparing recent data with those previously obtained in specific operations or even on flock level.

Any deviation from an established baseline is an important indicator in a strategic mycoplasma control programme, allowing conclusions about the overall mycoplasma field pressure and on the vaccination status.

Fig. 2. demonstrates how the titre of MG is slowly rising throughout the production cycle of a live vaccinated layer flock. 30 samples were taken, with varying CV levels. Presented is the GMT value.

The rise beyond a 3.000 titre value coincided in this case with the first appearance of MG disease signs in parts of the flock and a drop in egg production.

Fig. 2. The Titre of MG rising throughout the production cycle of a live vaccinated layer flock.



Titre values will vary depending on many factors, such as kit supplier, vaccination status, challenge levels in the field etc.

As such they always need to be interpreted with care as viewing them as fixed threshold can be misleading. This illustrates how the triad of diagnostic evidence, production data and disease presentation act together in correct disease diagnostics.

Rapid plate agglutination

The rapid plate agglutination (RPA) technique is based on the agglutination property of a known antigen in conjunction with an unknown antibody.

RPA is often used as a good screening technique for negative flocks. However, positive results usually need to be confirmed by another method.

Hemagglutination Inhibition test

The Hemagglutination Inhibition (HI) method is at least in theory less sensitive, but more specific than RPA and ELISA. One disadvantage is that it requires more time to perform and some more experience to do so correctly. ■

Conclusion

Conclusions from positive diagnostic methods as described will lose meaningfulness if there is no disease and production data available from the field to help understand the complete disease context.

A correct diagnosis relies on diagnostic methods or titres as described. It also relies on clinical signs of disease and the status of production data. All these aspects need to be considered in order to understand the impact of mycoplasma on production.

The described techniques can be used to evaluate the effectiveness of planned interventions and strategic control programs. Interventions are usually aimed at the improvement of production data, but success is also commonly evaluated by the decline of the mycoplasma load (refer to the qPCR box) as well as the evolution in ELISA titres. To determine the success of any intervention, key performance indicators need to be carefully selected beforehand.