



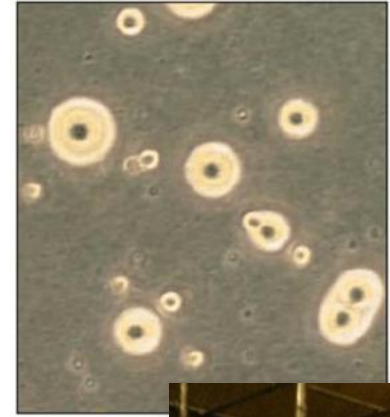
# Mycoplasma Monitoring

Key Consideration when using  
ELISA and PCR

Jantina De Vylder

# Avian Mycoplasma

- About 25 avian Mycoplasma species described
- Most important Mycoplasma spp for commercial chickens:
  - Mycoplasma gallisepticum (Mg)
  - Mycoplasma synoviae (Ms)
- One of the smallest self-replicating organism – No cell wall
- Attach and colonize mucosal surfaces
- Infection of respiratory, reproductive and musculoskeletal systems
- Transmitted horizontally and vertically
- Wide variation in virulence
- Antigenic variability



# Control of Avian Mycoplasma

- To prevent/reduce vertical and horizontal transmission of Mg/Ms:
  - Prevention
  - Vaccination
  - Antibiotics
- **Key to success** in preventing and controlling Mycoplasma is the use of an **effective monitoring program** in combination with the applied control strategy

# Diagnostic Tools

- **Serological Detection**

- Serum Plate Agglutination (or Rapid Plate Agglutination)
- Enzyme Linked ImmunoSorbent Assay
- Hemagglutination Inhibition Test



Antibody  
Detection

- **Molecular Detection**

- qPCR (+ Sequencing)
- DIVA qPCR



Pathogen  
Detection

- **Isolation + Identification (Culture)**

# Diagnostic Tools: General Considerations

- Temporal Sensitivity → Time Lag Serological Assays

- Initial response is IgM

- Detectable in 5-10 days
- RPA (or SPA)

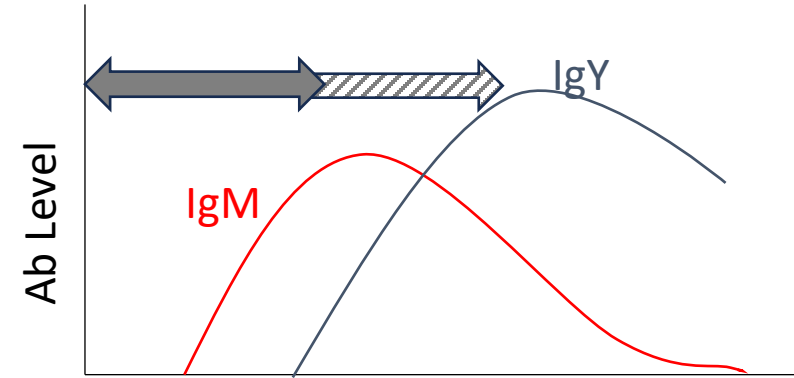
- IgY response follows

- Detectable after 2-3 weeks
- **ELISA** and HI

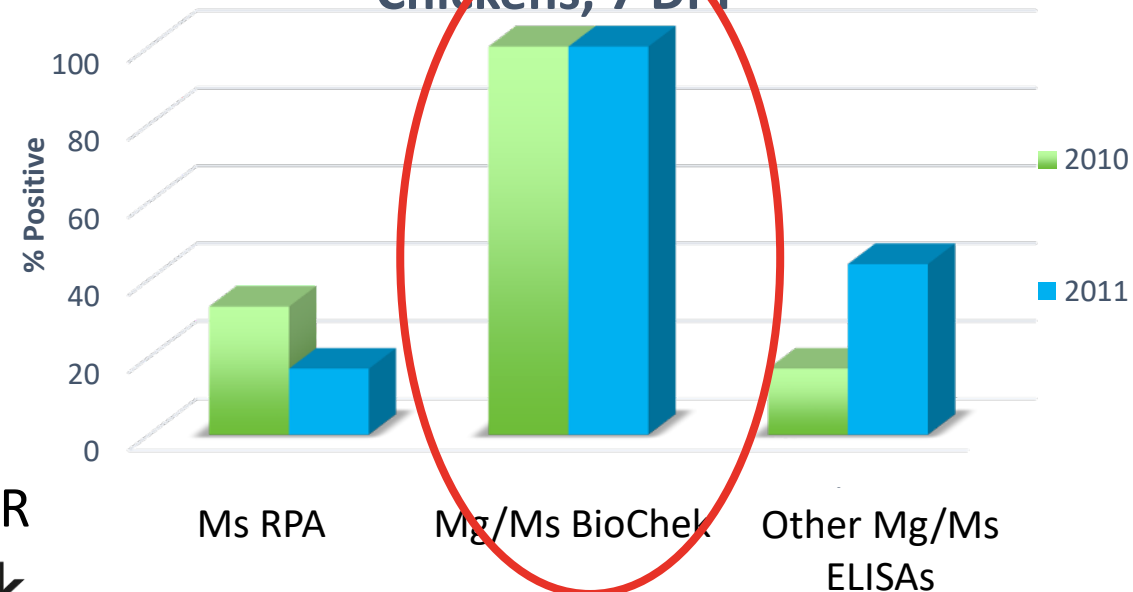
- Exception to above → BioChek MgMs ELISA detect antibodies as early as 7 days post-infection

→ Excellent temporal sensitivity

- Time Lag → Moving birds/Spiking males → PCR



MS Field Isolate in 4 Wk Old SPF Chickens, 7 DPI\*

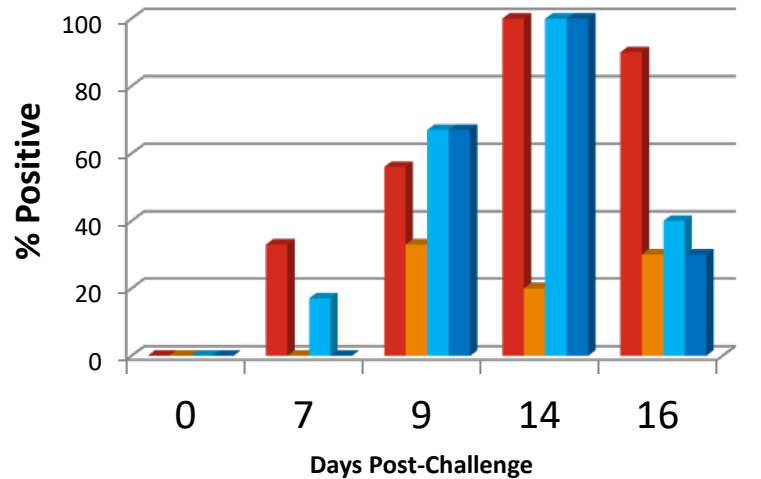


International PTS for Mg and Ms Report; Deventer Animal Health Services Ltd., Netherlands

# Diagnostic Tools: General considerations

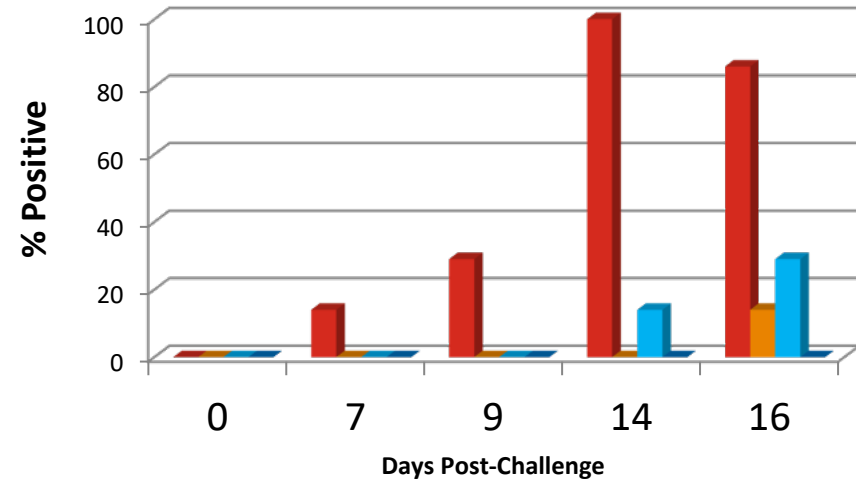
- Sensitivity – Typical Strain

MS WVU1853 Inoculated



■ BioChek MgMs ELISA ■ Mg/Ms (c) ELISA ■ RPA (1&2) ■ RPA (3)

MS WVU1853 Contacts



■ BioChek MgMs ELISA ■ Mg/Ms (c) ELISA ■ RPA (1&2) ■ RPA (3)

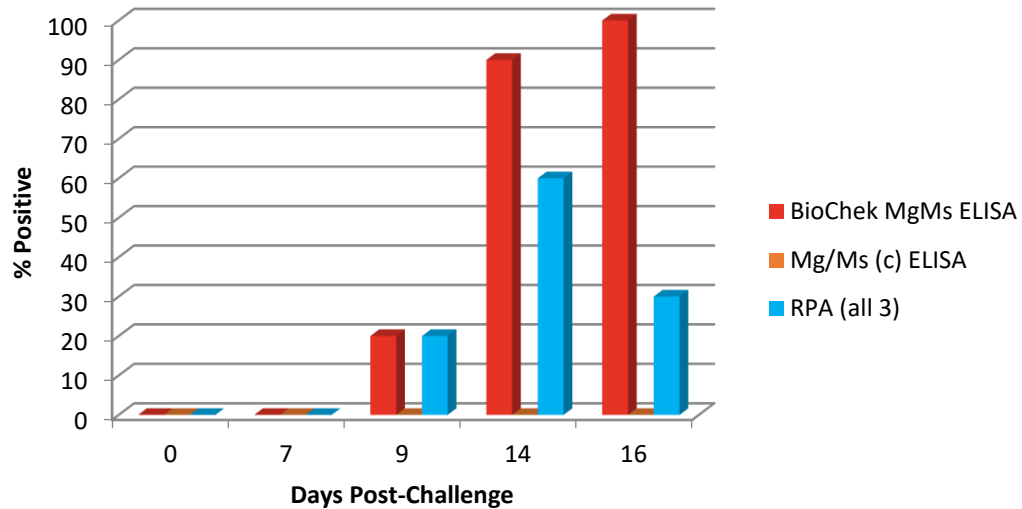
## PCR Results

Ms strain	Group	0 dpi	7 dpi	9 dpi	14 dpi
WVU1583	Infected	0/5	5/5	5/5	5/5
	Contact	0/5	4/5	5/5	5/5
K5664	Infected	0/5	5/5	5/5	5/5
	Contact	0/5	5/5	5/5	5/5

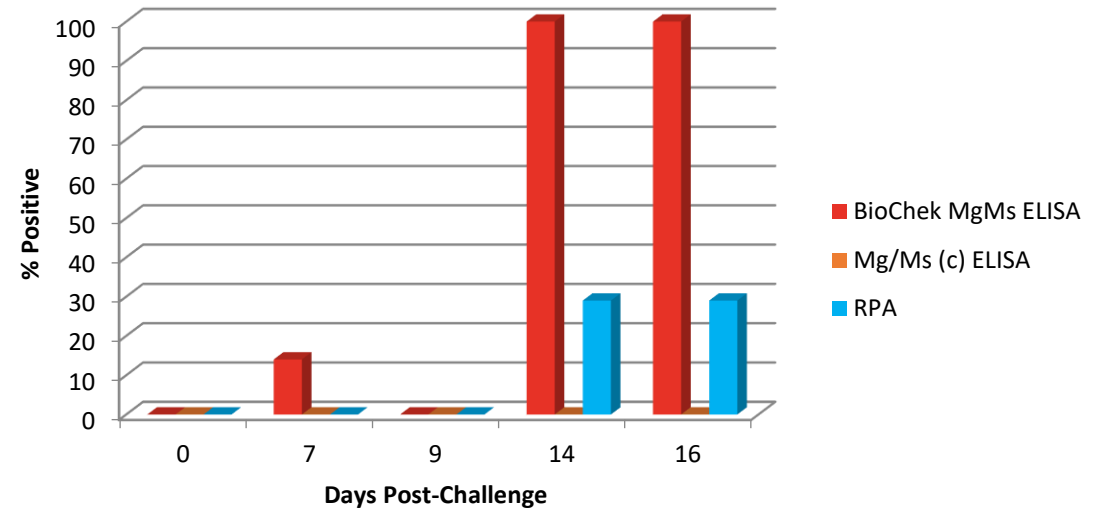
# Diagnostic Tools: General considerations

- Sensitivity – Atypical Strain

MS K56664 Inoculated



MS K56664 Contacts



## PCR Results

Ms strain	Group	0 dpi	7 dpi	9 dpi	14 dpi
WVU1583	Infected	0/5	5/5	5/5	5/5
	Contact	0/5	4/5	5/5	5/5
K5664	Infected	0/5	5/5	5/5	5/5
	Contact	0/5	5/5	5/5	5/5

# Diagnostic Tools: General considerations

- Specificity - False positives (Non-specific reactions - Cross-reactions) reactions can occur occasionally in any serological assay

Sera tested on RPA and commercial MgMs, Mg and Ms ELISAs (from 3 different suppliers) from SPF birds infected with *M. gallinarum*, *M. imitans*, *M. gallinaceum* in early stage and later stage post infection.

Period p.i.	<i>M. gallinarum</i>				<i>M. imitans</i>				<i>M. gallinaceum</i>				NC		SH			
	I <sup>1</sup>	Sp% <sup>4</sup>	II <sup>2</sup>	Sp%	I	Sp%	II	Sp%	I	Sp%	II	Sp%	I <sup>3</sup>	Sp%	II	Sp%	I	Sp%
Number of samples	n = 20		n = 20		n = 20		n = 20		n = 20		n = 20		n = 55		n = 10		n = 20	
RPA Mg <sup>5</sup>	2 <sup>ab6</sup>	90	0 <sup>a</sup>	100	3 <sup>ab*</sup>	85	0 <sup>a</sup>	100	0 <sup>a</sup>	100	0 <sup>a</sup>	100	0 <sup>a7</sup>	100	0 <sup>ab</sup>	100	0 <sup>a</sup>	100
RPA Ms <sup>5</sup>	6 <sup>a</sup>	70	0 <sup>a</sup>	100	6 <sup>a**</sup>	70	0 <sup>a</sup>	100	3 <sup>a</sup>	85	0 <sup>a</sup>	100	1 <sup>a</sup>	98	0 <sup>a</sup>	100	0 <sup>a</sup>	100
Mg ELISA A, B and C	0 <sup>b</sup>	100	0 <sup>a</sup>	100	0 <sup>b</sup>	100	0 <sup>a</sup>	100	0 <sup>a</sup>	100	0 <sup>a</sup>	100	0 <sup>a</sup>	100	0 <sup>a</sup>	100	n.t. <sup>9</sup>	
Ms ELISA A, B and C	0 <sup>b</sup>	100	0 <sup>a</sup>	100	0 <sup>b</sup>	100	0 <sup>a</sup>	100	0 <sup>a</sup>	100	0 <sup>a</sup>	100	0 <sup>a</sup>	100	0 <sup>a</sup>	100	n.t.	
Mg/Ms combi ELISA A	0 <sup>b</sup>	100	0 <sup>a</sup>	100	0 <sup>b</sup>	100	0 <sup>a</sup>	100	0 <sup>a</sup>	100	0 <sup>a</sup>	100	0 <sup>a</sup>	100	0 <sup>a</sup>	100	n.t.	
Mg/Ms combi ELISA B	0 <sup>b</sup>	100	0 <sup>a</sup>	100	0 <sup>b</sup>	100	1 <sup>a***</sup>	95	0 <sup>a</sup>	100	0 <sup>a</sup>	100	0 <sup>a</sup>	100	0 <sup>a</sup>	100	n.t.	
Mg/Ms combi ELISA C	0 <sup>b</sup>	100	0 <sup>a</sup>	100	0 <sup>b</sup>	100	0 <sup>a</sup>	100	0 <sup>a</sup>	100	1 <sup>a</sup>	95	0 <sup>a</sup>	100	0 <sup>a</sup>	100	n.t.	

Feberwee et al, 2020

Number of false positives with RPA (in different groups) >> ELISAs

# Diagnostic Tools: PCR

- To overcome some of the limitations of serological assay
- High Specificity/Sensitivity
- vetproof *Mycoplasma Gallisepticum-Synoviae* qPCR LyoKit

## ➤ 4-plex

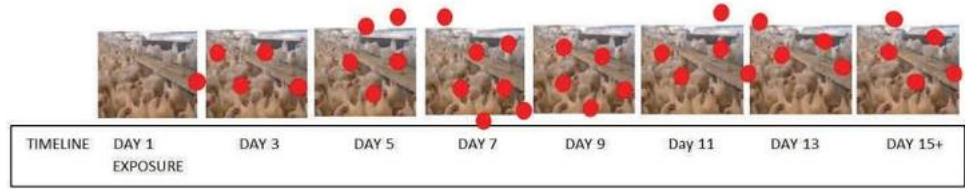
- *Mycoplasma Gallisepticum*
- *Mycoplasma Synoviae*
- Endogenous control → avian DNA → Verify sample quality, extraction and amplification
- Internal (amplification) control

## ➤ Lyophilized format

- Into the tubes
- Only need to add sample (25 µl per rxn)

➔ Unique Dual Control System → Reliable results with all sample types

➔ Unique Format → Less handling errors - Less contamination risk



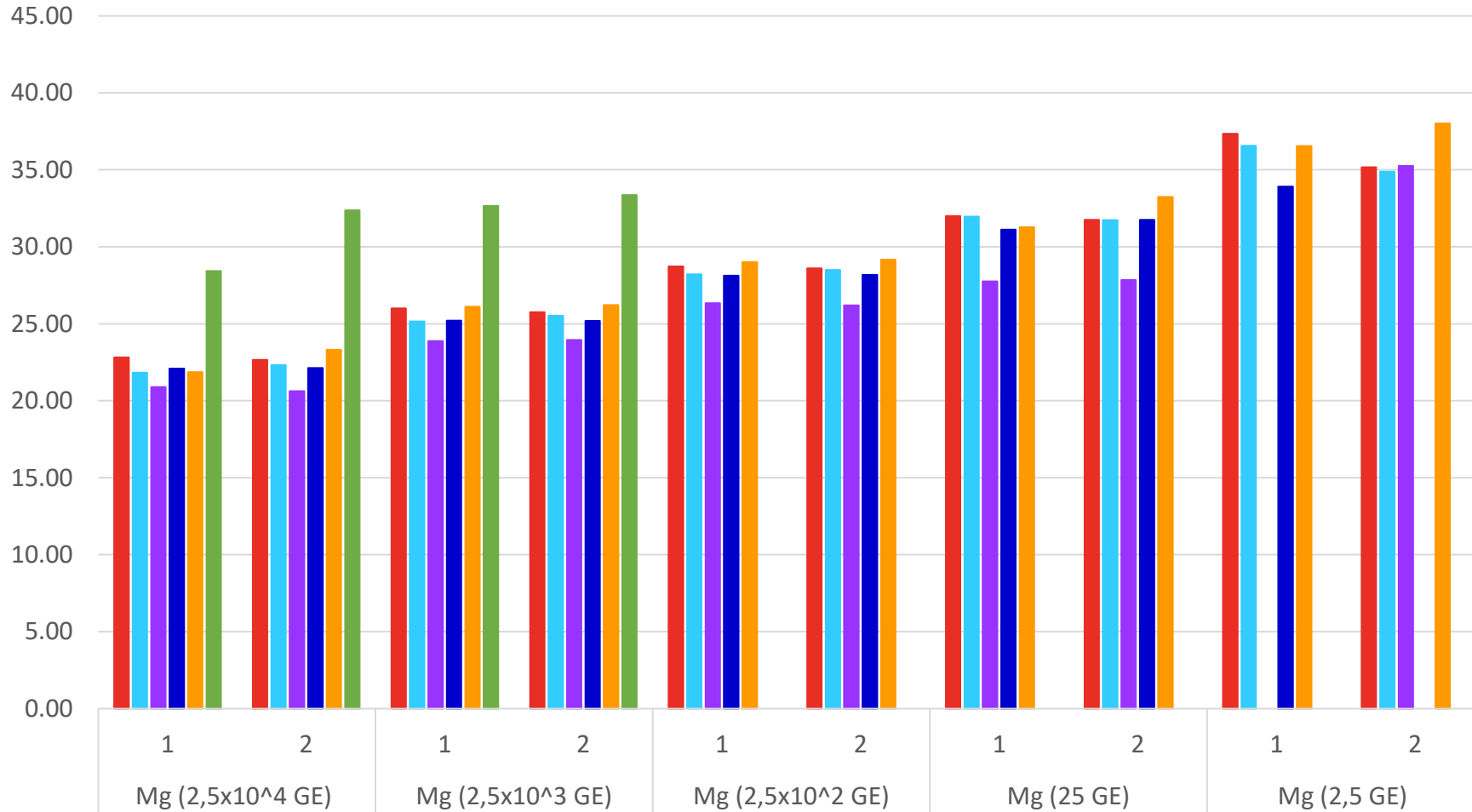
PCR	NEG	POS	POS	POS	POS	POS	POS	POS
ELISA	NEG	NEG	NEG	POS	POS	POS	POS	POS
HI	NEG	NEG	NEG	NEG	NEG	POS	POS	POS

Provided by B. Glidewell - GPLN



# Diagnostic Tools: General considerations

Dilution Serie of Mg DNA with background Ms DNA (5x10<sup>4</sup> GE)



All samples tested in duplo

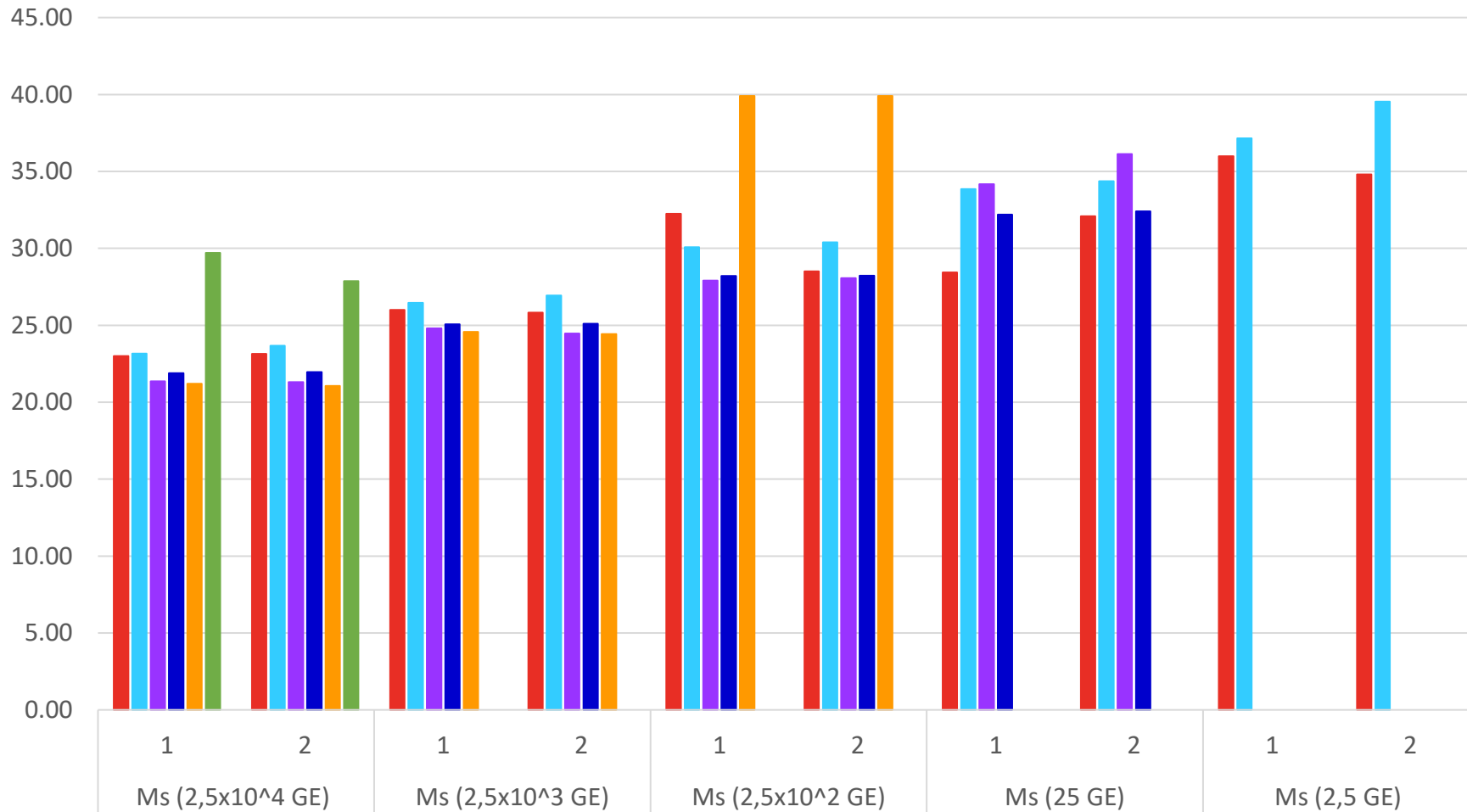
Only 5 µl of sample was used on the vetproof MgMs qPCR LyoKit (comparability)

All PCRs detected the background Ms DNA in all samples

- vetproof MgMs qPCR LyoKit
- Competitor 1
- Competitor 2
- Competitor 3
- Competitor 4
- WOAH (Raviv & Kleven)

# Diagnostic Tools: General considerations

Dilution Serie of Ms DNA with background Mg DNA ( $5 \times 10^4$  GE)



All samples tested in duplo

Only 5  $\mu$ l of sample was used on the vetproof MgMs qPCR LyoKit (comparability)

All PCRs detected the background Mg DNA in all samples

- vetproof MgMs qPCR LyoKit
- Competitor 1
- Competitor 2
- Competitor 3
- Competitor 4
- WOAH (Raviv & Kleven)

# Diagnostic Tools: General considerations

- PCR/Extraction Method - Inhibition

- Swabs from chickens – Mg & Ms – PBS – Heat boil extraction
- Same sample extract – Tested in duplo – 6 PCR

Real-time PCR	Mg	Ms	IC/EC
vetproof MgMs qPCR LyoKit	Pos/Pos	Pos/Pos	✓/✓
Competitor A	Pos/Pos	Pos/Pos	✓/✓
Competitor B	Neg/Neg	Neg/Neg	x/x
Competitor C	Pos/Pos	Pos/Pos	✓/✓
Competitor D	Neg/Neg	Neg/Neg	✓/✓
WOAH (Raviv & Kleven)	Neg/Neg	Neg/Neg	N/A

Internal and/or External  
(endogenous or exogenous)  
Amplification Control

Control indicated  
**inhibition** → Result invalid

IC did not indicate problem  
No IC

**!!! FALSE NEGATIVES !!!**

→ Importance of using a test with proper controls and running in an internal validation

# Diagnostic Tools: General considerations

- PCR/Extraction Method - Inhibition
  - Swabs from chickens – Mg & Ms – Magnetic Bead Extraction
  - Same sample extract – Tested in duplo – 6 PCRs

Real-time PCR	Mg	Ms	IC/EC
vetproof MgMs qPCR LyoKit	Pos/Pos	Pos/Pos	✓/✓
Competitor A	Pos/Pos	Pos/Pos	✓/✓
Competitor B	Pos/Pos	Pos/Pos	✓/✓
Competitor C	Pos/Pos	Pos/Pos	✓/✓
Competitor D	Pos/Pos	Pos/Pos	✓/✓
WOAH (Raviv & Kleven)	Neg/Neg	Neg/Neg	N/A

Internal and/or External  
(endogenous or exogenous)  
Amplification Control

No IC / Sensitivity?

!!! FALSE NEGATIVES !!!

→ Importance of using a test with proper controls and running in an internal validation

# Diagnostic Tools: General considerations

- Specificity PCR – Inclusivity & Exclusivity

Genus	Species	Origin/Strain	Mg	Ms	IC	EnC
Mycoplasma	gallisepticum	NCTC 10115	+	-	+	-
Mycoplasma	gallisepticum	ATCC 15302	+	-	+	-
Mycoplasma	gallisepticum	Nobilis® Mg 6/85	+	-	+	-
Mycoplasma	gallisepticum	Poulvac® Myco F	+	-	+	-
Mycoplasma	gallisepticum	Vaxsafe® Mg (TS-11)	+	-	+	-
Mycoplasma	gallisepticum	FLI Mg R30	+	-	+	-
Mycoplasma	gallisepticum	FLI Mg 231	+	-	+	-
Mycoplasma	gallisepticum	FLI Mg 17/90	+	-	+	-
Mycoplasma	gallisepticum	FLI Mg 340/97	+	-	+	-
Mycoplasma	gallisepticum	FLI Mg 16DL0144 AL	+	-	+	-
Mycoplasma	synoviae	NCTC 10124	-	+	+	-
Mycoplasma	synoviae	ATCC 25204	-	+	+	-
Mycoplasma	synoviae	H 11253A/1e 33C	-	+	+	-
Mycoplasma	synoviae	FLI Ms 345	-	+	+	-
Mycoplasma	synoviae	FLI Ms 14 DL	-	+	+	-
Mycoplasma	synoviae	FLI Ms 1853	-	+	+	-

- 72 other poultry micro-organism, incl non-target Mycoplasma strains

- **No false positive detection of Mg nor Ms**

# Diagnostic Tools: ELISA & PCR

- ELISA & PCR are powerful tools for monitoring Mycoplasma in poultry flocks worldwide due to:
  - Good sensitivity & specificity
  - Quick time to results
  - Handle large sample volumes efficiently
- Both methods have their unique advantages and disadvantages. Understanding the limitations is key when setting up a successful Mycoplasma monitoring program for your flocks in line with the applied control measures.

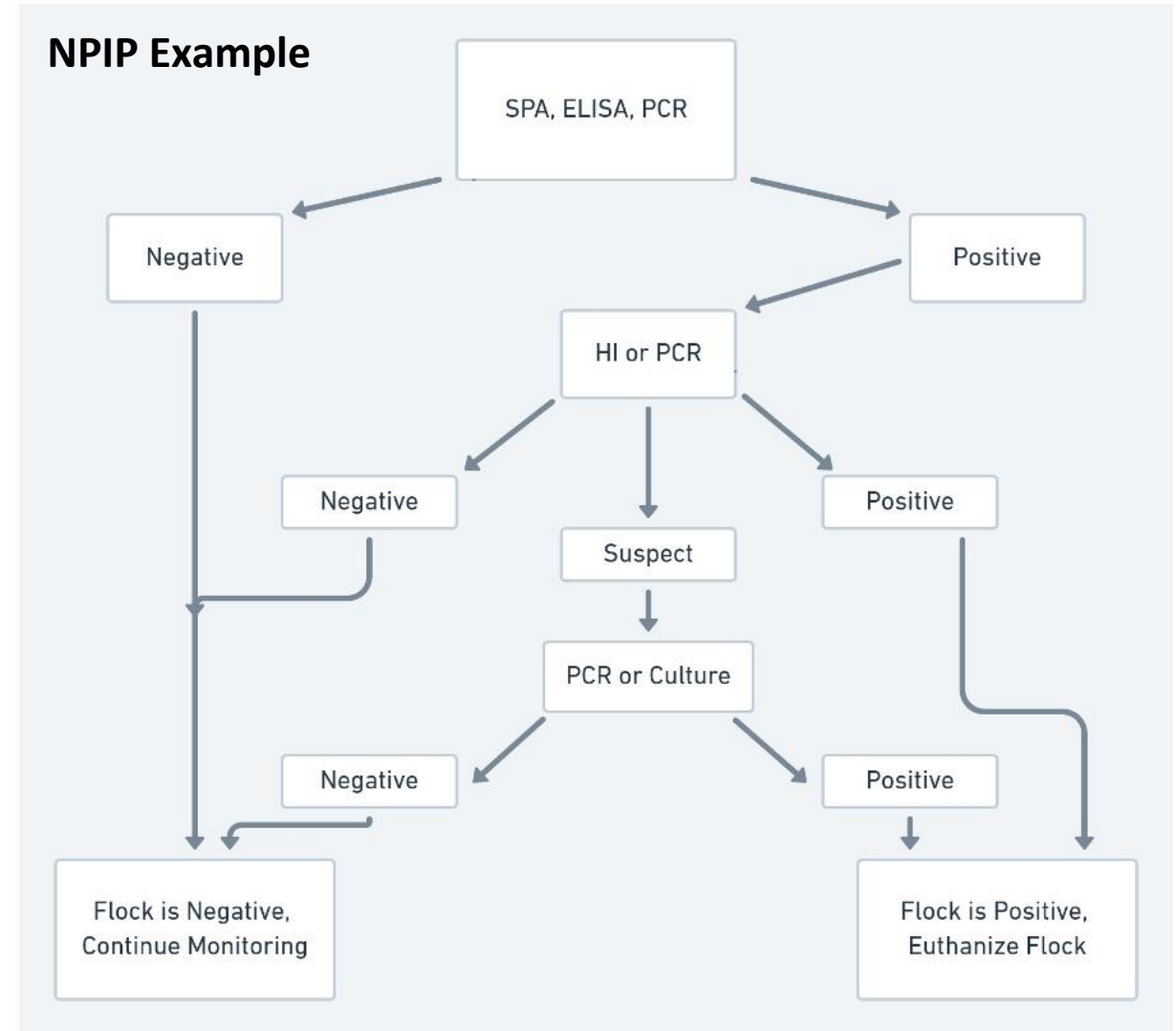
# Control Strategy 1: Prevention

## Monitoring in non-vaccinated flocks:

- The ideal test is 100% accurate & precise and 100% sensitive & specific
- Test should be highly sensitive (early detection of infection/ no false negatives)
  - Temporal (detects infection in early stages)
  - Strain (detects all strains)
- Test should be highly specific (no false positives)
  - Less work (no retesting)
  - Lower costs (no retesting)
- Both ELISA and PCR are suitable test for use in a monitoring program

# Control Strategy 1: Prevention

- Good consistent monitoring program:
  - Screening test
  - Confirmatory test plan
  - Takes into account sample size & frequency



# Control Strategy 1: Prevention/Elimination

Non-vaccinated Broiler Breeder Flock – 51 weeks of age – Routine Monitoring → **Early Infection**

House #	MgMs ELISA Quant	MgMs ELISA Titers	HI Results	PCR 2 wks later	New samples 3 wks later	ELISA Results HI Results PCR Results
<b>HS 2 - M</b>	3/3 Pos	2647 2483 2589	MG & MS Neg		HS 2 M & F	<p><u>200 serum samples HS 2</u>  <b>199 + ELISA:</b> 1 @ 353;            199 @ 794-11362            MG HI Neg  <b>153 MS HI +:</b> 33 Neg; 75@20;            51@40; 19@80; 6@160; 2@320;            14 Invalid</p> <p><b>PCR MS +</b>            PCR MG -</p>
<b>HS 2 - F</b>	4/12 Pos	2022 1973 2009 1916	MG & MS Neg	<b>MS PCR +</b> MG PCR -	HS 2 M & F	

# Control Strategy 1: Prevention/Elimination

- Monitoring: Alternating scheme ELISA & PCR



!!! PCR is the preferred test when moving birds !!!

# Control Strategy 2: Vaccination

- Use of vaccines makes monitoring more complicated:
  - Most tests (ELISA/PCR) will become positive (to a certain degree)
  - Decide if result is positive due to:
    - Vaccination
    - Field Challenge
    - Or both
- Live (conventional) vaccines
  - Induce serological response (! varying levels !)
  - Interferes with PCR → DIVA assay/sequencing required
- Recombinant vaccine (FP vector)
  - Serological response absent
  - No interference with PCRs
- Inactivated vaccines
  - Induce serological response (declining titers and seropositivity level over time!)
  - No interference with PCR

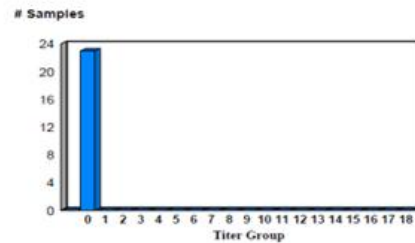
# Control Strategy 2: Vaccination

- Live (conventional) vaccines

		Weeks post vaccination				
		4	10	20	30	36
% PCR positives	6/85 aerosol	35	50	50	80	42
	ts-11 eye drop	84	70	20	0	5

Noormohammadi & Whithear, 2019

Age : 48W  
 Type : BB  
 House No. : 03  
 Reason for Testing : CONTROL  
 Vaccination Program : BA



Assay : Mg Lot: FS5638  
 Bleeding Date : 04-08-2013 Testing Date : 07-10-2013

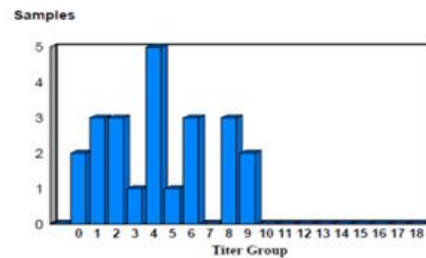
Mean Titer: 60  
 Min. - Max Titer : 1 - 195  
 G.M.T.: 33  
 %CV : 88

VI Index: 1

**BB vaccinated with 6/85  
 At 06W and 14w with atomist  
 spray**

At 48 weeks birds negative as expected

Age : 56W  
 Type : BB  
 House No. : 03  
 Reason for Testing : CONTROL  
 Vaccination Program : BA



Assay : Mg Lot: FS5672  
 Bleeding Date : 26-09-2013 Testing Date : 07-10-2013

Mean Titer: 4932  
 Min. - Max Titer : 437 - 13 519  
 G.M.T.: 3101  
 %CV : 87

VI Index: 57

At 56 weeks birds  
 Serology positive (MT = 4932, 91%  
 seropositivity)  
 Indicative for a WT challenge  
 No clinical symptoms  
 Birds seem to be protected

Details Vaccination Program:

Vaccine	Method	Age	Vacc.Batch/Applicator
6/85	fine spray	06W	
6/85	fine spray	14W	/

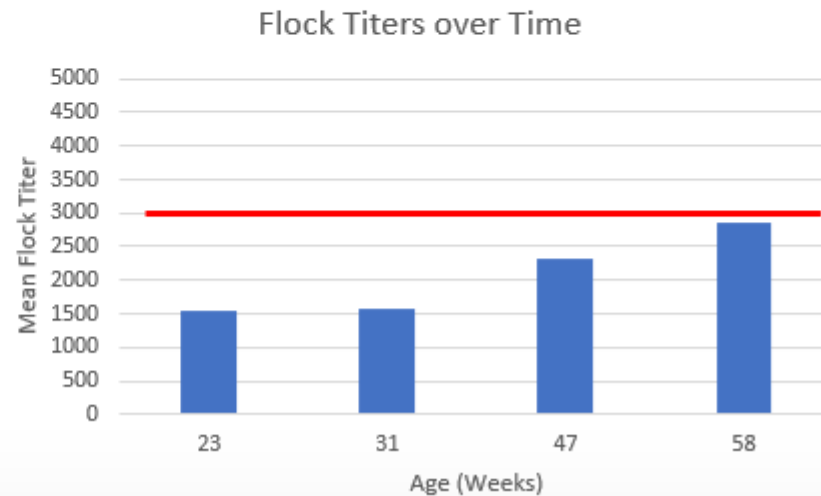
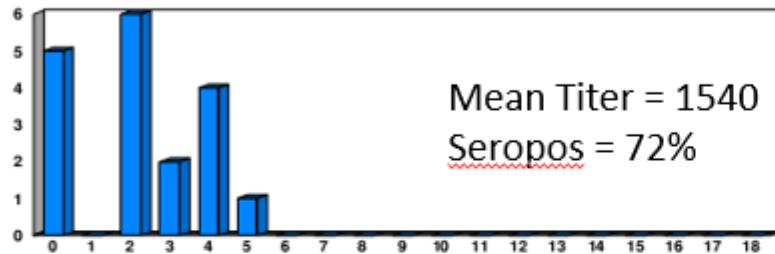
Titer Range Ref. Controls: 10 (1000-4000)  
 Mean Titer Ref. Controls: 10=2646

Positive Cutoff S/P >= 0.50 Total No. Samples: 23 Neg/Sus/Pos = 2 / 0 / 21

# Control Strategy 2: Vaccination

- Live (conventional) vaccines

Broiler Breeder Flock – Age 23 weeks  
Vaccinated with MS-H at 18 weeks of age



**Mg/Ms PCR: MS positive**

**Further molecular analysis → Only vaccine strain (MS-H) detected**

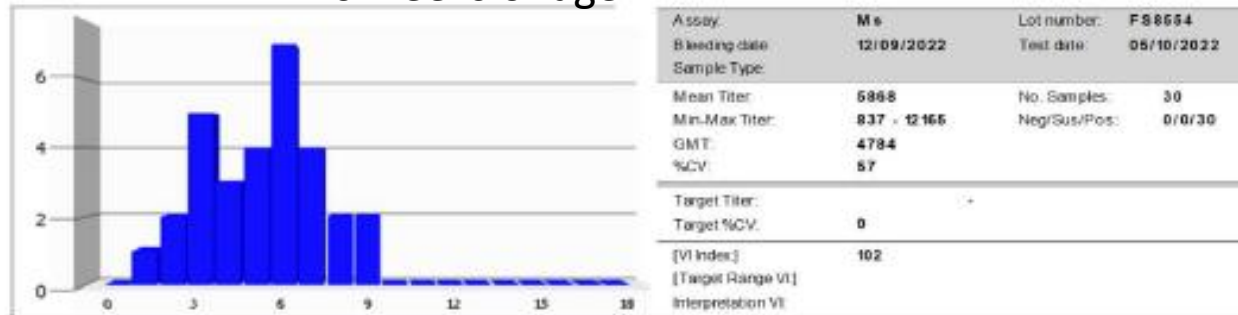
# Control Strategy 2: Vaccination

- Inactivated vaccine

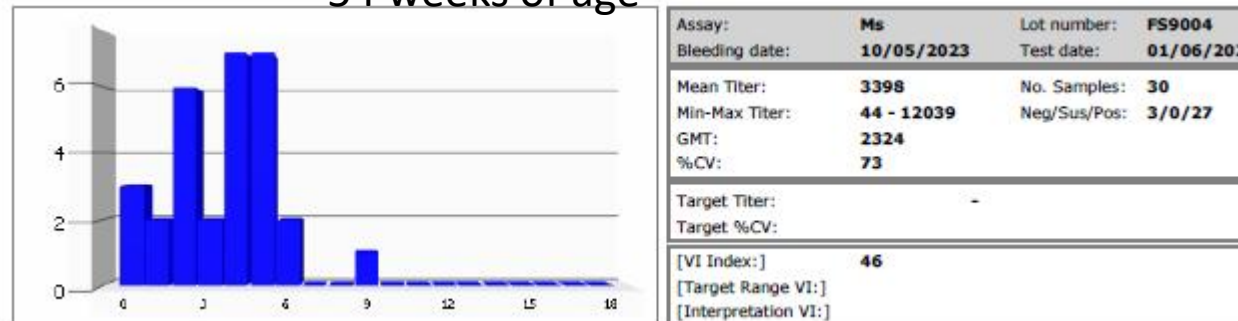
Broiler Breeder Flock

Vaccinated with Ms Inac at 10 and 14 weeks of age

20 weeks of age

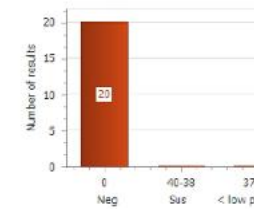


54 weeks of age



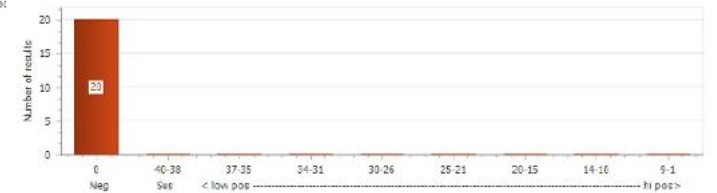
Assay:	Vetproof SI Mycoplasma Multiplex Detection kit	Lot Number:	
Target:	<b>Mg</b>	Matrix:	Tracheal Swab
Sampling Date:	06/10/2022 00:00:00	Test Date:	06/10/2022 12:20:50

Cq results



Assay:	Vetproof SI Mycoplasma Multiplex Detection kit	Lot Number:	
Target:	<b>Ms</b>	Matrix:	Tracheal Swab
Sampling Date:	06/10/2022 00:00:00	Test Date:	06/10/2022 12:20:50

Cq results



PCR –

PCR → No Mg/Ms detected

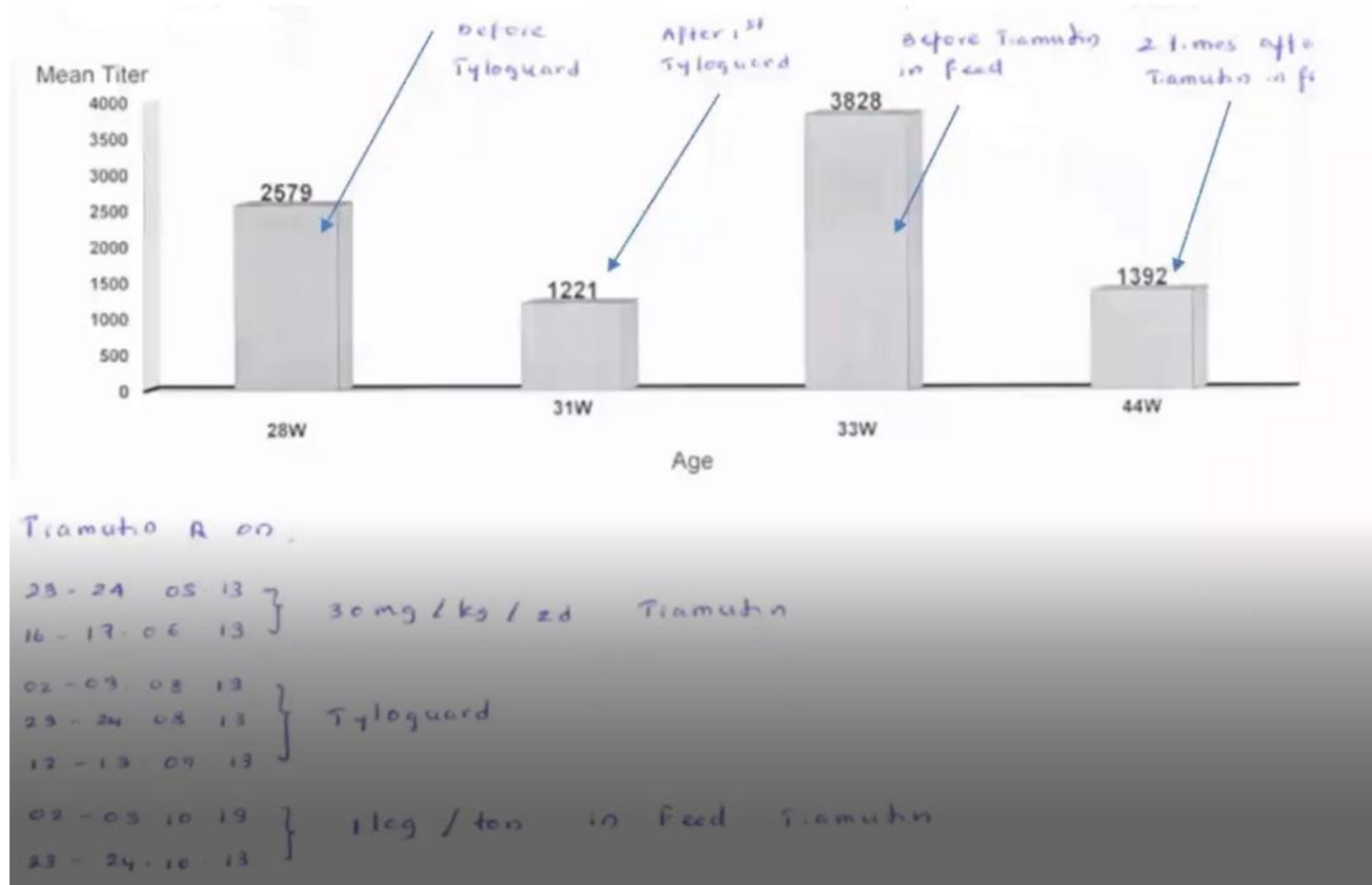
Positive serology due to vaccination

## Control Strategy 2: Vaccination

- Depending on the vaccine, route of administration, number of applications and timing between vaccination and sampling, variable antibody levels can be seen
  - ELISA results after vaccination require careful interpretation
  - Know what can be expected
  - Significant increases in titers (*and level of seropositivity*) are indicative for challenge
  - Inconclusive results/challenging interpretation requires further testing
- PCR → Conventional live Mg and Ms vaccines
  - After vaccination → Positive PCR → Further testing required to differentiate if PCR is positive because of vaccine and/or field

# Control Strategy 3: Antibiotics

- Antibiotic treatment - ELISA

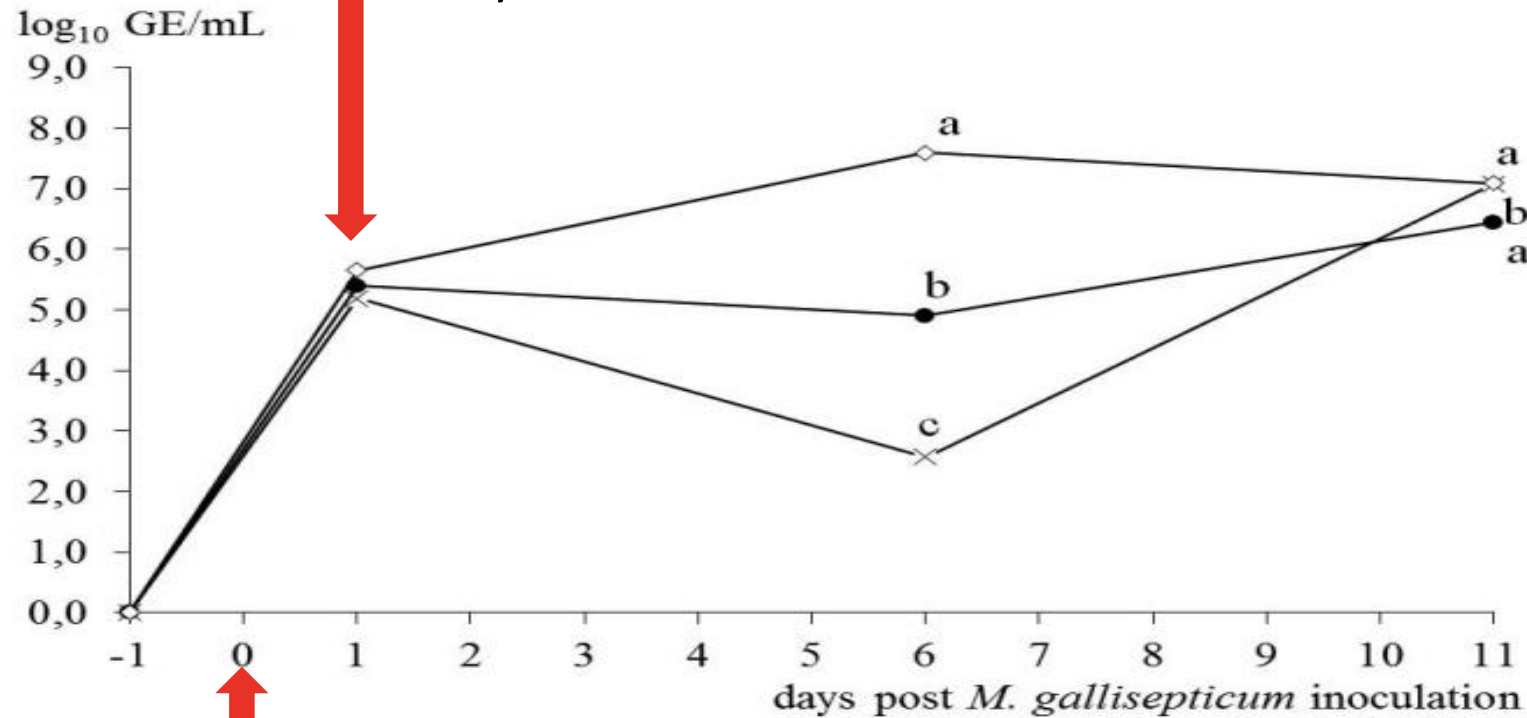


Antimicrobial treatment against Mycoplasma can influence ELISA titers!  
→ Monitor over time

# Control Strategy 3: Antibiotics

- Antibiotic treatment – Tracheal swabs – qPCR Results

Start tylosine treatment via drinking water for 5 consecutive days



- ◇ Control Group
- 35 mg tylosin/kg BW/day
- x 100 mg tylosin/kg BW/day

Antimicrobial treatment against Mycoplasma impacts PCR results!  
→ Monitor over time

Intratracheal inoculation broilers (10 days of age) with Mg field isolate

# Conclusion

Vaccinated	Type of vaccine	Infected	Serology		Detection of Pathogen	
			Screening	Confirmation	Screening or Confirmation	DIVA/Seq required
No	None	No	Neg	(Neg)*	Neg	No
		Yes	Pos	Pos	Pos	No
Yes	Inactivated	No	Pos	N/A*	Neg	No
		Yes	Pos	N/A*	Pos	No
	Live Conventional	No	Neg/Pos**	N/A*	Neg/Pos	Yes
		Yes	Pos**	N/A*	Pos	Yes
Live Vector	No	Neg	N/A*	Neg	No	
	Yes	Pos	N/A*	Pos	No	

\* Not necessary

\*\* Serology results may be inconclusive

Adapted from A. Feberwee et al, 2022

# Conclusion

- ELISA and PCR are powerful tools that can be applied in Mycoplasma monitoring programs
- Every tests has specific characteristics, benefits and limitations
- Select the right assay(s) for monitoring your Mycoplasma control strategy

Thank you!

