



Avian Mycoplasma Situation in Egypt

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Incidence of MG by PCR in 49 commercial chicken flocks

Aim

- (1) To study the incidence of MG in broiler, layer and breeder flocks in 3 Egyptian governorates**
- (2) To differentiate between F vaccine and MG field strains using Restriction Fragment Length Polymorphism (RFLP of pvpA gene)**

Method

1- Semi nested PCR

Two amplifications were performed on two steps;
amplification of pvpA gene

then amplification of C-terminus-encoding region of the same gene

2- RFLP assay of pvpA gene C-terminas encoding region

Semi nested PCR
Amplification of the *pvpA* C-terminus-encoding region

Initial primers were designed from the *pvpA* gene of R strain Boguslavsky *et al.*, 2000

Primer 1 (*pvpA1F*), located at nucleotide positions 415 to 437 (5'GCC AMT CCA ACT CAA CAA GCT GA3EER

Primer 2 (*pvpA2R*), located at nucleotide positions 1059 to 1081 (5'GGA CGT SGT CCT GGC TGG TTA GC3

Primer 3 (*pvpA3F*) located at nucleotide positions 583 to 604 (5'GGT AGT CCT AAG TTA TTA GGT C3')

AccuOligo[®], BION

The PCR positive band ranged between **350-410 bp** after the 2nd amplification

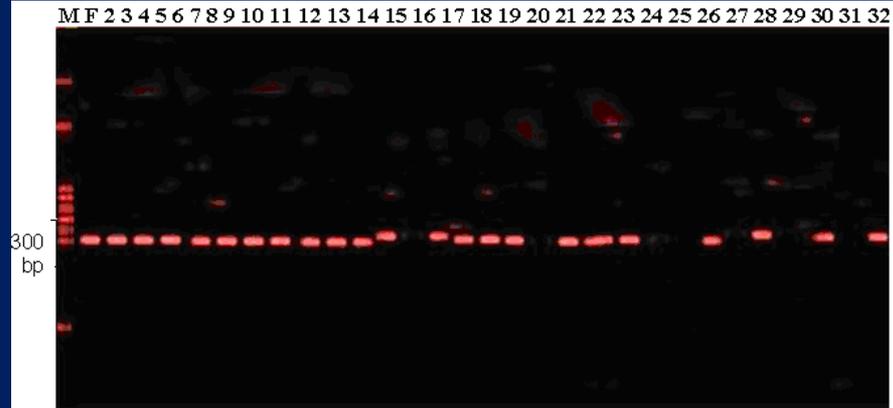


Photo (1): PCR of field samples (lanes 2-32); lane M: 100 bp DNA marker (Fermentas# SM0243), lane F: F strain vaccine.



Photo (2): PCR of field samples; Lane 1: 100 bp DNA ladder (Fermentas# SM0243). Lane 2 forward, field samples. (33-47). Lane F: F vaccine.

Incidence of MG in 49 commercial chicken flocks by PCR

Breed	positives/total
Layer, Breeder, Broiler Chickens	37/49 75.5%

RFLP was performed with **FastDigest® PvuII**
enzyme (Fermentas, #FD0634)

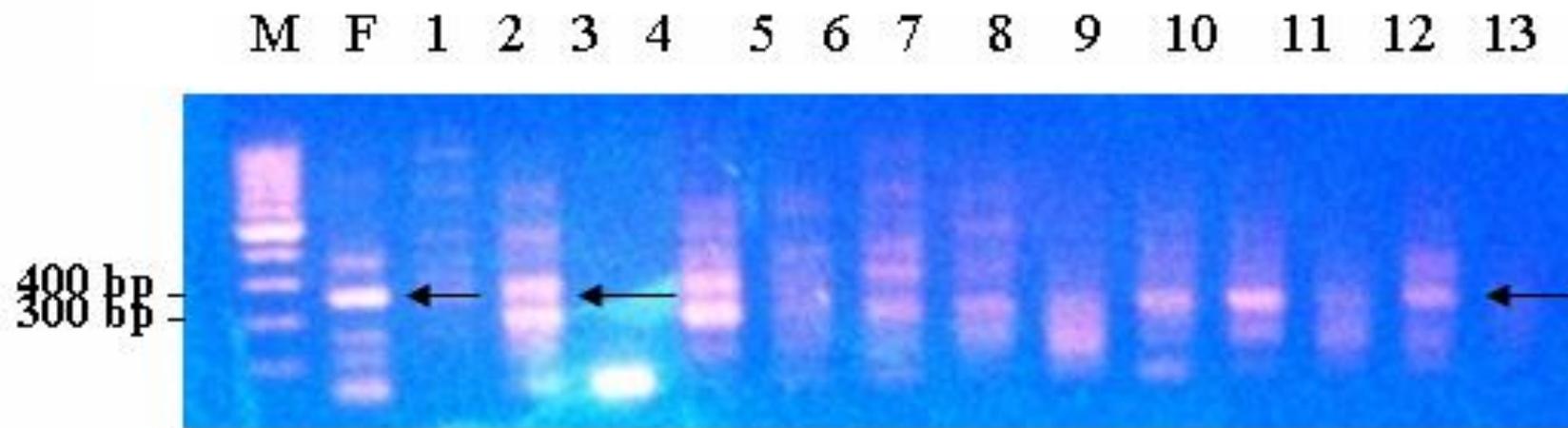


Photo (4): PCR of experimental samples. Lane 1, DNA Marker; lane 2, F vaccine; Lane 3 & forward, experimental samples.

RFLP data were analyzed by **Total Lab. Software Analysis version 1.1**
Cluster Analysis was done by **PAST version 1**

Ladder F 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35

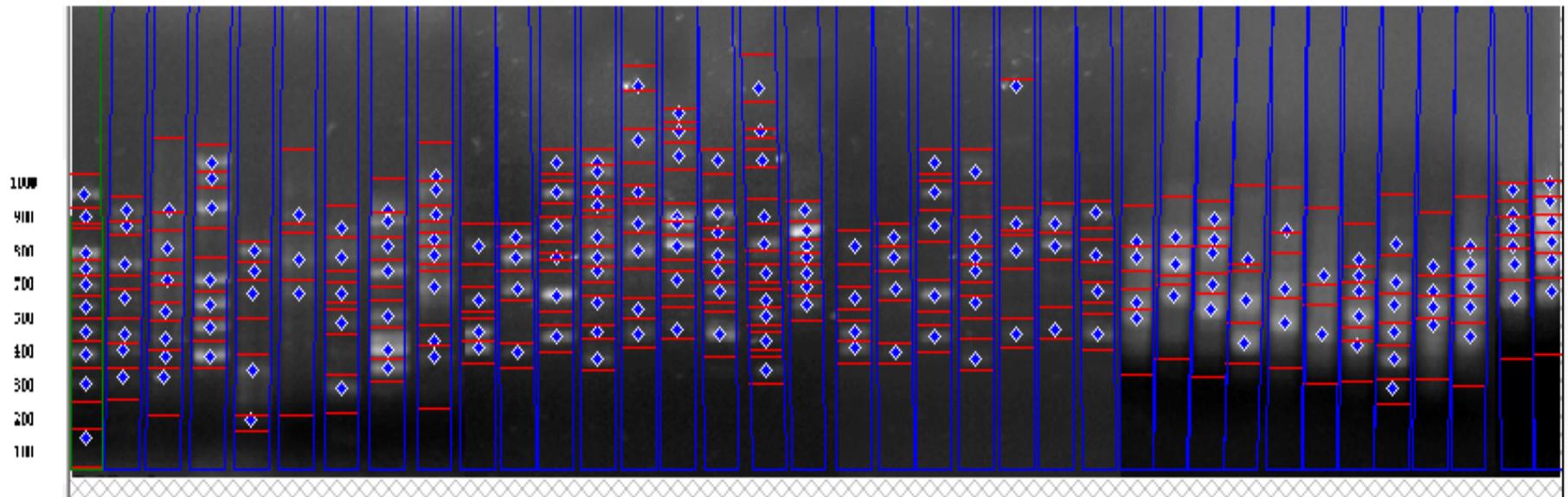


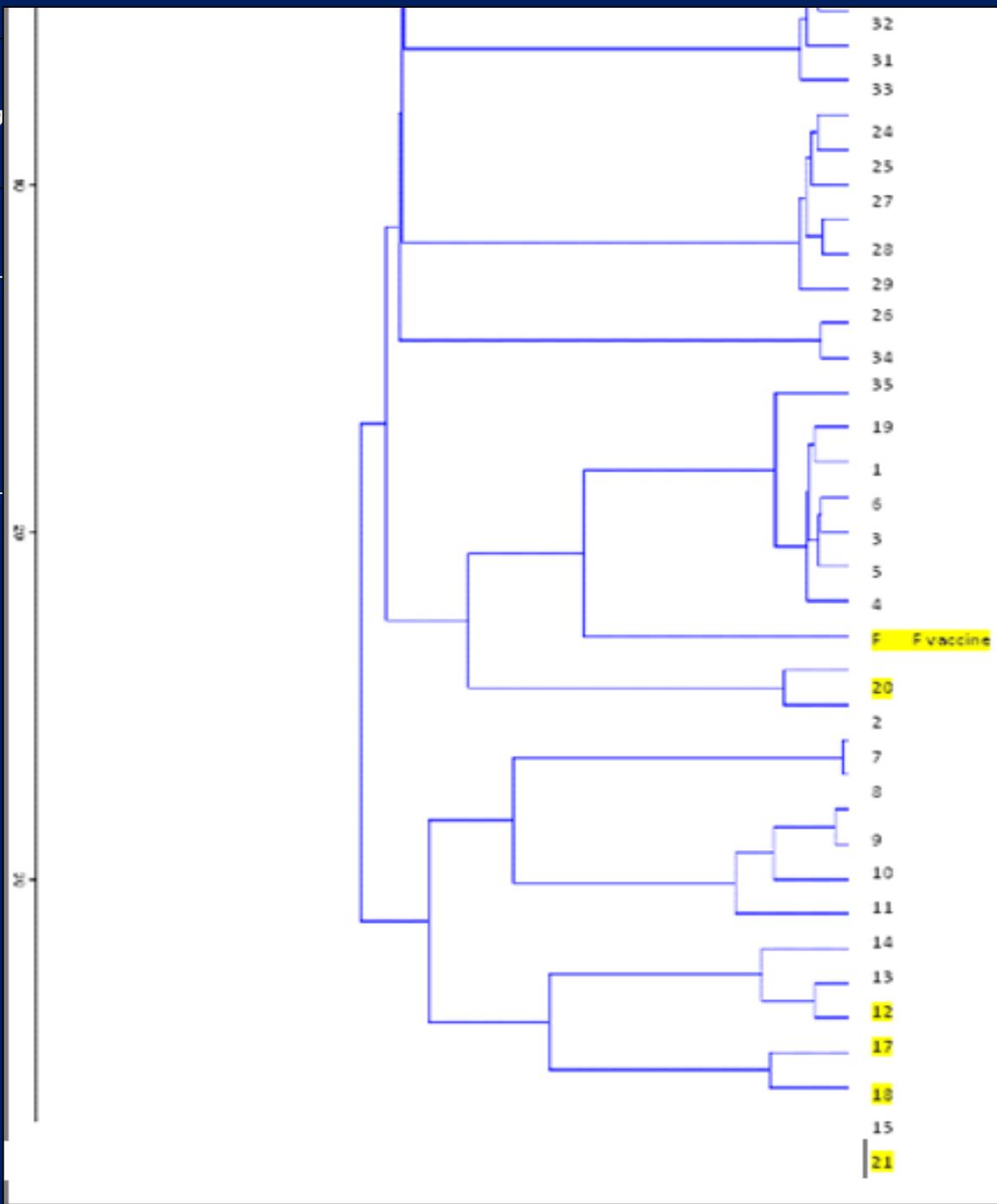
Photo (3): Computerized analysis for RFLP analysis for C-terminous region of pvpA for 35 positive PCR samples and F vaccine.

Lane 1, DNA Ladder (Fermentas# SM0243); Lan 2, F vaccine.

**Phylogenic analysis of
the C-terminus-encoding portion of *p*
based on RFLP results.**

6 MG isolates (no. 1, 3, 4, 5, 6 & 19) were more related to **MG-F vaccine** strain with more than **92%** genetic similarity.

2 MG isolates (no. **9 & 28**) were both from one layer flock, one isolate obtained at **6 days** of age while the other was recovered at **25 wks** of age, but they showed **38%** relatednes.



Conclusions

a- Identity among MG isolates ranged between 38% to up to 90% using RFLP

b- 6 isolates were similar to F vaccine strain by 90-93%

Experiment

Aim

A-To compare between the pathogenicity and transmissibility of 3 selected F vaccine like strains - **MG** field isolates with that of F-strain vaccine

- **Experimental design**

- A total number of 220-one-day-old Mg and Ms parent, male chicks were divided into 6 groups:
- 220 of one-day-old Groups 1, 2, 3; contained 120 chicks (40 in each, where 20 were infected with 3 individual isolates no. 23, 36 and 37 respectively + 20 x 3 chicks in each group were left in contact to the infected mates in the same battery section).
- Group 4; contained 40 chicks (20 chicks were vaccinated with single dose of F strain vaccine + 20 chicks were left in contact in the same battery section).
- Group 5; included 40 chicks (20 were vaccinated with double dose of F strain vaccine + 20 chicks were left in contact in the same battery section).
- Group 6; contained 20 chicks kept as non vaccinated, non infected control group in a separate battery.

Shedding of MG in trachea of experimental chickens by pooled PCR (number of positive /total tested samples)

Groups	Age			
	2wks		4 wks	
	infected	Contact	Infected	Contact
infected with isolate 1	4/4	3/4	0/4	1/4
infected with isolate 2	4/4	3/4	4/4	2/4
infected with isolate 3	0/4	0/4	4/4	2/4
4 (one dose vaccine)	0/4	3/4	4/4	2/4
5 (double dose vaccine)	4/4	2/4	4/4	2/4
6	0/4		0/24	

ELISA Serological Response of Experimental Chickens at 4 & 5 wks PI

	4 wks PI		5 wks PI	
Group	Infected	Contact	Infected	Contact
1 infected with isolate 1 (F strain 93% identity)	0/7	1/7	0/7	1/7
2 infected with isolate 2	0/7	0/7	0/7	0/7
3 infected with isolate 3 (F strain 93% identity)	0/7	0/7	0/7	0/7
4 One dose of F-vaccine	1/7 low titer	0/7	0/7	0/7
5 Double dose of F-vaccine	1/7 high titer 2/7 low titer	0/7	2/7	0/7
6 Non infected, no contact	0/7		0/7	

Age(day)	Groups	Clinical signs		
		Conjunctivitis	Airsacculitis	Pneumonia
Group 1				
14 th day	Infected	-	+	-
	Contact	-	+	-
21 st day	Infected	+		
	Contact	-		
28 th day	Infected	+	-	-
	Contact	-	+	-
Group 2				
14 th day	Infected	-	+	+
	Contact	-	+	+
21 st day	Infected	+		
	Contact	-		
28 th day	Infected	+	-	-
	Contact	-	+	-
Group 3				
14 th day	Infected	-	+	-
	Contact	-	-	-
21 st day	Infected	+		
	Contact	-		
28 th day	Infected	+	-	-
	Contact	-	+	-
Group 4				
14 th day	Vaccinated	-	-	-
	Contact	-	-	-
21 st day	Vaccinated	-		
	Contact	-		
28 th day	Vaccinated	-	-	-
	Contact	-	-	-
Group 5*				
14 th day	Vaccinated	-	-	-
	Contact	-	-	-
21 st day	Vaccinated	-		
	Contact	-		
28 th day	Vaccinated	+++	-	-
	Contact	+++	-	-
Group 6				
14	Non	-	-	-
21	vaccinated	-		
28	non infected	-	-	-

Respiratory signs and PM lesions of experimental birds infected with different MG isolates and F vaccine

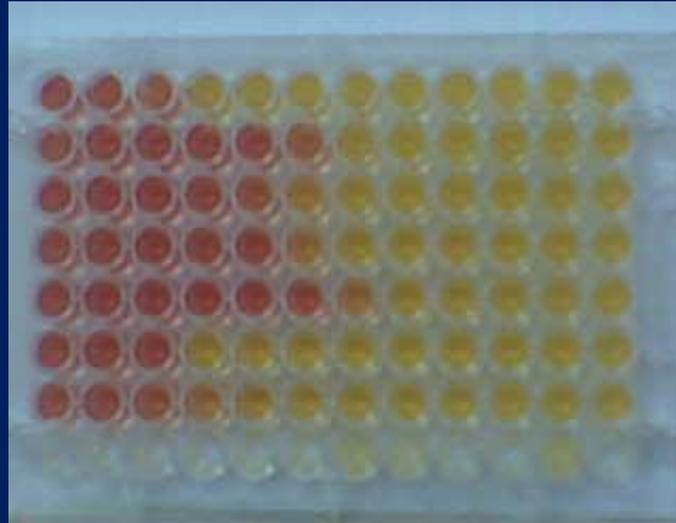
Conclusion

1-F vaccine like strains of MG when inoculated experimentally induced mild conjunctivitis and airsacculitis lesions in infected but not in contact chickens

2-They also were very weak in induction of noticeable serological response until 5 weeks PI either in infected or contact

3-They appeared in shedding from the trachea in majority of experimental chickens after 2 weeks of infection or contact and still at high rate of shedding up to 4 weeks PI even in contact

4-This was similar to the behaviour of F vaccine but there was no pathogenicity of this F vaccine in infected or contact chickens



MG Antibiotic Sensitivity Pattern

(MIC₉₀)

μg/ml 2005-2008

Isolate	Cipro.	Tylosin	Linco/spect	Tiamuli.	Doxy	Enro.	Erythro.
1	0.4	0.025	-	0.0125	0.05	0.0125	≥ 6.4
2	6.4	0.2	-	0.05	0.003	0.1	≥ 6.4
3	6.4	0.025	-	0.4	0.006	0.05	3.2
4	1.6	0.2	-	0.05	0.4	3.2	0.8
5	1.6	0.2	0.05	0.05	0.0125	3.2	3.2
6	6.4	≥ 6.4	6.4	0.4	0.4	0.1	3.2
7	0.4	0.4	6.4	0.05	≥ 6.4	3.2	0.8
8	6.4	0.2		0.05	0.4	0.1	3.2
9	1.6	0.2	0.4	0.4	0.4	0.0125	3.2
10	0.2	0.4	1.6	0.4	0.05	≥ 6.4	≥ 6.4
11	0.06	0.04	0.08	0.002	0.04	0.05	0.08
13	0.04	0.01	0.01	0.02	0.05	0.04	0.08

(MIC₉₀) ug/ml 2010-2012 MG

Isolat es	Cipro.	Tylosin	Tiam / Chlorotetra	Tiamulin	Doxy.	Enro.	Erythro.
1	0.2	-	0.1	0.1	0.05	0.4	3.2
2	0.2	-	0.2	0.2	0.2	0.4	0.4
3	0.1	-	0.4	0.8	0.2	0.4	3.2
4	0.1	-	0.1	0.1	0.2	0.8	-
5	0.003	0.012	0.003	0.006	0.05	0.006	3.2
6	0.8	-	0.4	0.8	0.2	0.8	-
7	0.016	-	0.002	0.016	0.002	0.002	-
8	0.003	0.003	0.003	0.003	0.003	0.025	3.2
9	0.003	1.6	0.003	0.003	0.05	0.4	3.2
10	0.003	0.2	0.003	0.003	0.003	0.2	3.2
11	0.003	0.8	0.003	0.05	0.025	0.4	1.6
12	0.8	-	0.4	0.8	0.1	0.2	-
13	0.8	-	0.4	0.8	0.05	0.025	-
14	0.05	6.4	0.0125	0.025	0.0125	0.4	6.4

(MIC₉₀)
ug/ml 2014-2015 for MG

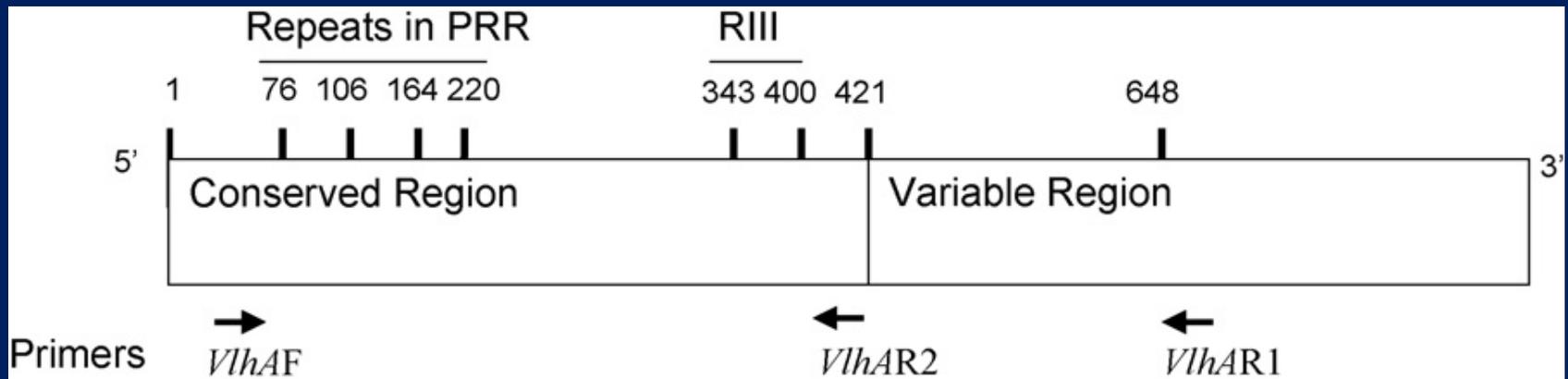
Isola tes	Cipro.	Tylosin	tilmicosin	linco/ spect	Norflox	Tiamulin	Doxy	Erythro
1	0.4	8	2	0.5	2	12.8	0.8	12.8
2	0.025	32	2	4	1	0.125	2	16
3	0.125	4	8	1	16	1	0.625	4
4	1	6.25	0.25	2	6.25	1	0.625	12.5
5	0.125	64	16	4	2	4	4	64
6	0.625	2	4	0.5	4	8	0.625	2
7	0.625	32	32	4	4	16	8	64
8	0.8	32	32	2	4	2	8	64
9	1	8	16	2	8	32	8	4

MS
Diagnosis

Detection of *MS* by PCR from Respiratory Tissues in regional local flocks during 2013

Noormohammadi *et al.*, (2000) said that the 5-*vlhA* region codon is present in the *Ms* genome as a single copy and does not change its sequence in clonal populations

Diagrammatic representation of the *Ms vlhA* gene (based on Bencina *et al.*, 2001) and the primer locations



*** Primers for PCR (0.025 umole):**

***VlhAF* Forward 5' ATTAGCAGCTAGTGCA GTGGCC 3'**

***VlhAR1* Reverse 5' CAGCGCTAGTTTTGTTTTTTGG 3'**

***VlhAR2* Reverse 5' AGTAACCGATCCGCTTAATGC 3'**

**MS
positive by PCR**

Breeder & Layers

Broilers

4 / 19

4 / 26

21.05%

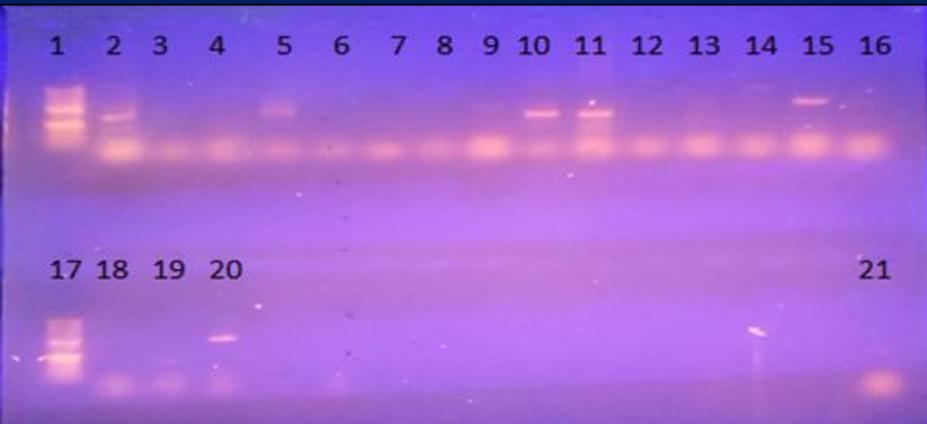
15.38%



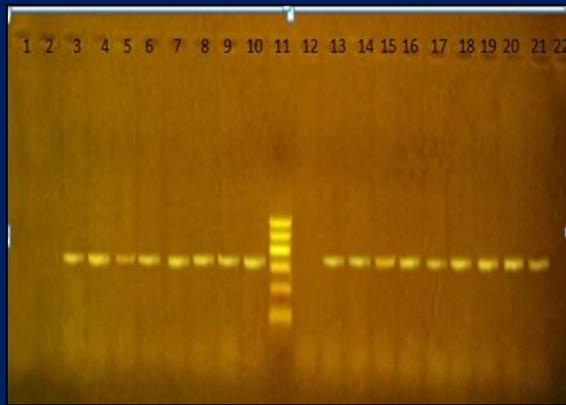
The 3 Egyptian strains of MS were not related to the strains from neighboring countries (Israel, Iran) but they were related to Japanese and Armenian and Brazilian ones

☞ Incidence of **MS** from both respiratory and arthritic problems In 3 neighboring Egyptian governorates (January – July 2015)

Type of chickens	positive flocks	Age of flock
Broilers	31	1day – 36 days of age
Layers & Breeders	9	3 weeks – 58 weeks

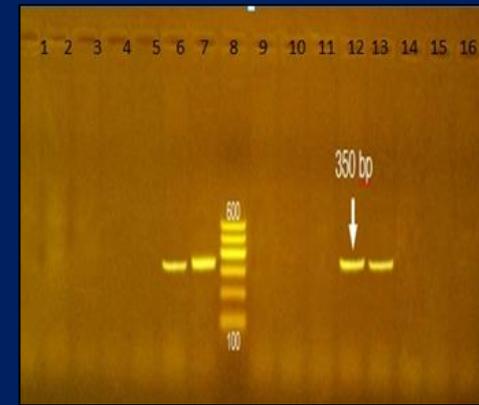


Lane 1 and 17: 50 bp DNA ladder, lanes 2-5-9-10-11 and 21 positive samples (350-400 bp) amplification of *M. synoviae vIhA* gene.



(a)

PCR - MS 2015



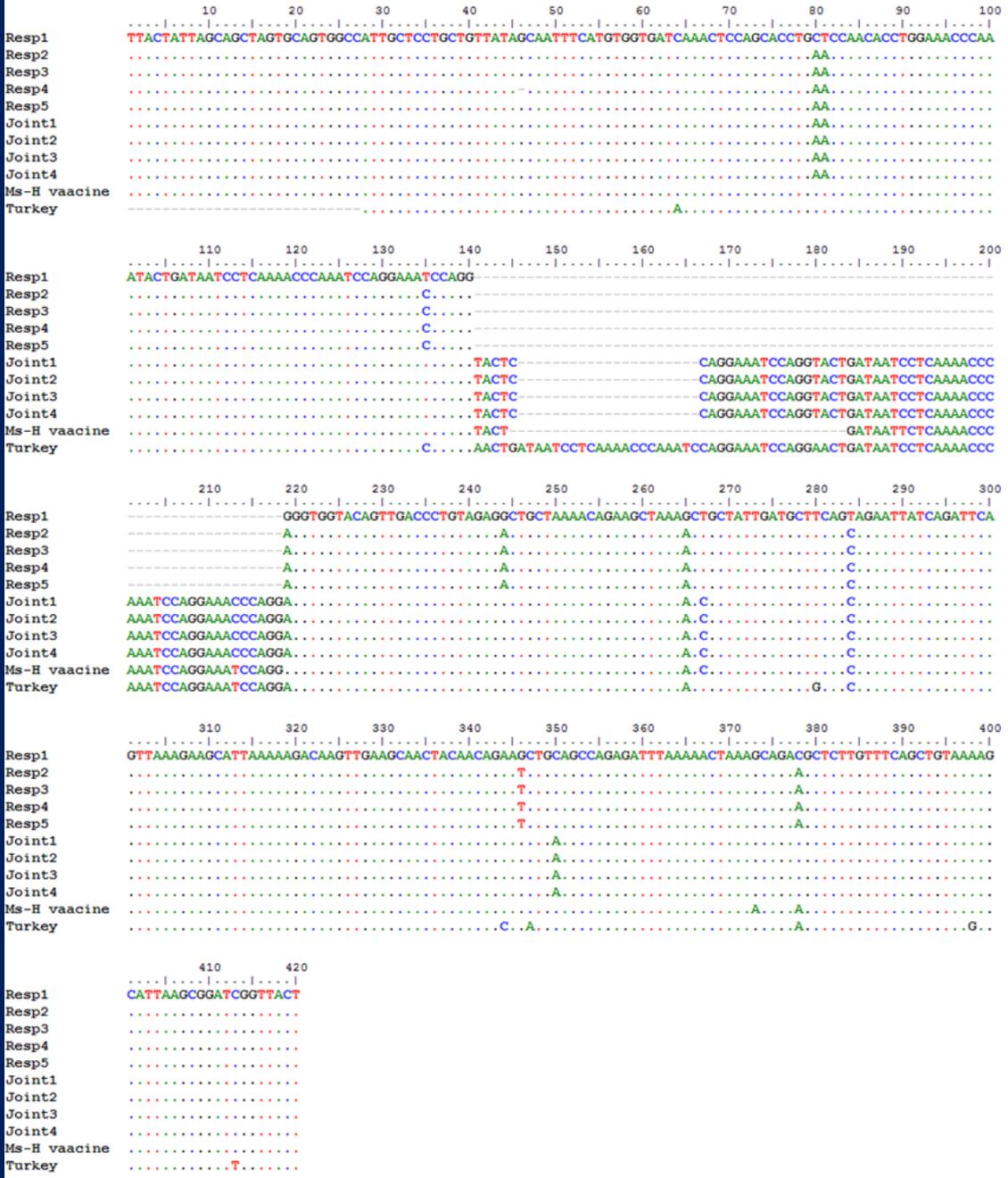
(b)

PCR electrophoresis gel demonstrating *M. synoviae vIhA* gene amplification isolated directly from synovial fluid. Lane 11, DNA molecular weight ladder (100 bp) in (a) and lane (8) in (b). Lanes (2,3,4,5,6,7,8,9,10,13,14,16,16,17,18,19,20,21) are positive for *M. synoviae* in (a) and lanes (6,7,12,13) are positive for *M. synoviae* in (b). Lanes (1,2,12) are negative for *M. synoviae* in (a) and lanes (1,2,3,4,5,9,10,11,14,15,16) are negative for *M. synoviae* in (b).

Incidence of *MS* from both respiratory and arthritic problems in 40 commercial chickens in neighboring Egyptian governorates (January – July 2015)

Type of chickens	positive flocks		Age of flock	Clinical signs & PM
Broilers	5/31		1day	Dead in shell
			27 days	CCRD, Avian influenza, Mortality
	2 / 5 arthritis	3 / 5 CCRD	28 days	Airsaccultis, Mortality
			32 days	CCRD, IB, ND
			33 days	Arthritis, CCRD
Layers & Breeders	5/9		3 weeks	Arthritis, CCRD,
			12 weeks	Arthritis, CCRD
	3 / 5 arthritis	2 / 5 CCRD	36 weeks	Arthritis, mortality
			42 weeks	CCRD, inflammation of oviduct
			58 weeks	CCRD

Comparison of the partial nucleotide sequence of *vlhA* gene of *M. synoviae*.



Isolate designation	Accession numbers in GenBank
EGY2.sqn EGY.Ras.resp.1	KT957960
EGY2.sqn EGY.Ras.resp.2	KT957961
EGY2.sqn EGY.Ras.resp.3	KT957962
EGY2.sqn EGY.Ras.resp.4	KT957963
EGY2.sqn EGY.Ras.resp.5	KT957964
EGY2.sqn EGY.Ras.joint.1	KT957965
EGY2.sqn EGY.Ras.joint.2	KT957966
EGY2.sqn EGY.Ras.joint.3	KT957967
EGY2.sqn EGY.Ras.joint.4	KT957968

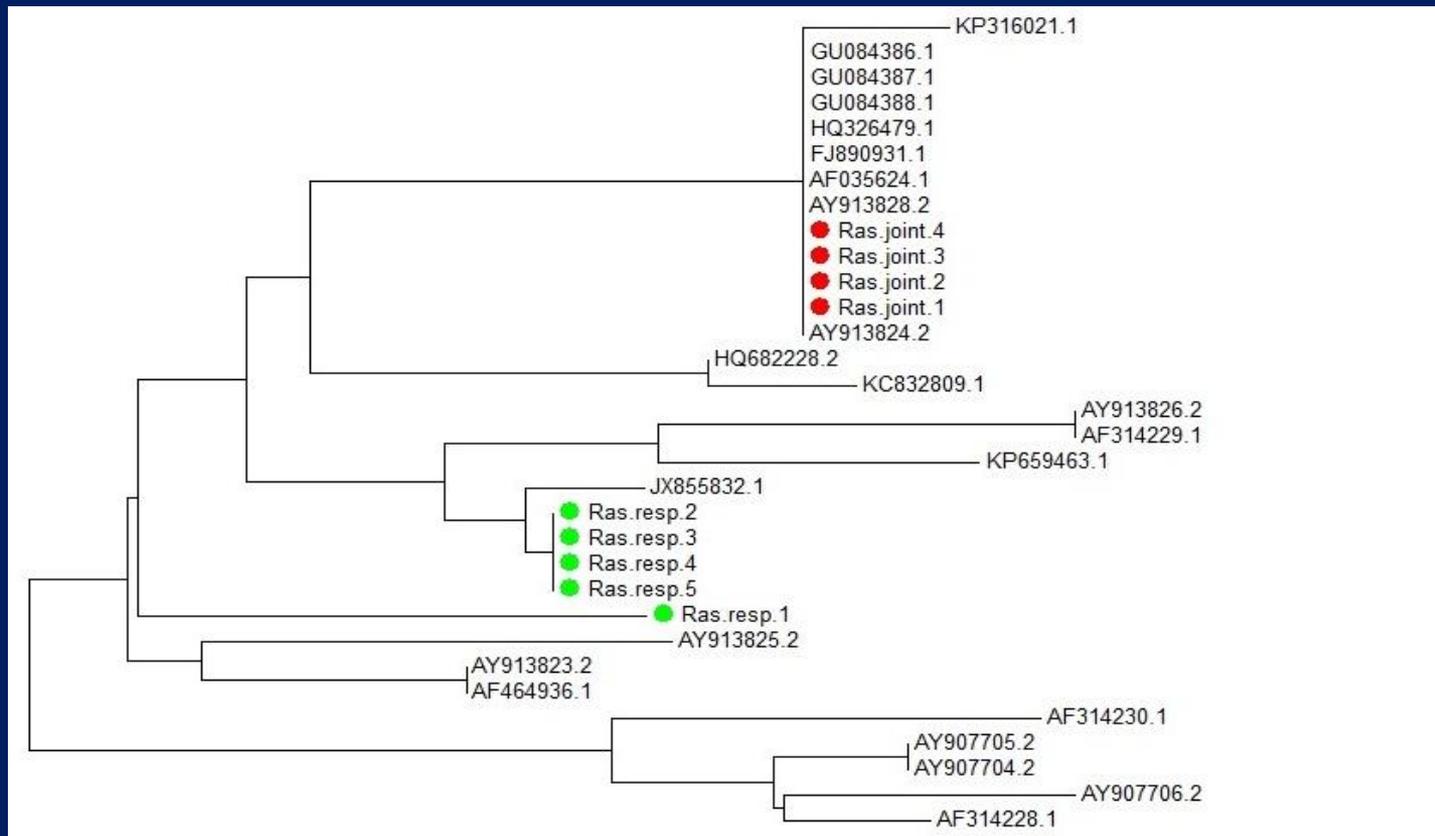
Regarding the partial *vlhA* gene sequences

All **joint** samples showed **complete similarity** in between

There was **92.9%** similarity between **vaccine MS-H** and **joint *M. synoviae*** isolates

There was **89.5%** similarity between **vaccine MS-H** and **respiratory *M. synoviae*** isolates

There was **85.5%** similarity between **joint *M. synoviae*** isolates and **respiratory *M. synoviae*** isolates.



Phylogenetic tree based on the partial sequence of *vIhA* gene of *M. synoviae*. The sequences were obtained from the 9 samples in this study and sequence from AF464936.1 (MS-H strain) and other sequencing of other strain from gene bank

Deletions and Insertions

- There was deletion of **57** nucleotides from **respiratory *M. synoviae*** isolates when compared with **joint *M. synoviae*** isolates.
- There was an insertion of **18** nucleotides in **joint *M. synoviae*** isolates when compared with **vaccine MS-H**.

		10	20	30	40	50	60
Resp1	LLLAASAVAIAPAVIAISCGDQTPAPAPPTPGNPNTDNPQNPNPGNP-----					
Resp2E.....					
Resp3E.....					
Resp4E.....					
Resp5E.....					
Joint1E.....GTP-----GNPG					
Joint2E.....GTP-----GNPG					
Joint3E.....GTP-----GNPG					
Joint4E.....GTP-----GNPG					
Ms-H vaccineGT-----					
TurkeyK.....GTDNPQNPNPGNPG					

		70	80	90	100	110	120
Resp1	----- ----- ----- ----- ----- ----- -----	GGGTVDPVEAAKTEAKAAIDASVELSDSVKEALKRQVEATTTEAAARD					
Resp2	----- ----- ----- ----- ----- ----- -----T.....T.....A.....S.....					
Resp3	----- ----- ----- ----- ----- ----- -----T.....T.....A.....S.....					
Resp4	----- ----- ----- ----- ----- ----- -----T.....T.....A.....S.....					
Resp5	----- ----- ----- ----- ----- ----- -----T.....T.....A.....S.....					
Joint1	TDNPQNPNPGNP.....T.....A.....E.....					
Joint2	TDNPQNPNPGNP.....T.....A.....E.....					
Joint3	TDNPQNPNPGNP.....T.....A.....E.....					
Joint4	TDNPQNPNPGNP.....T.....A.....E.....					
Ms-H vaccine	-DNSQNPNPGNP.....T.....A.....					
Turkey	TDNPQNPNPGNP.....T.....AA.....AD.....					

		130	140
Resp1	LKTKADALVSAVKALSGSVT	
Resp2E.....	
Resp3E.....	
Resp4E.....	
Resp5E.....	
Joint1	
Joint2	
Joint3	
Joint4	
Ms-H vaccineTE.....	
TurkeyE.....R.....L.....	

Comparison of the amino acid sequences of the N-terminal region of MSPB proteins predicted from the 5'-end *v/hA* sequences of 9 *M. synoviae* isolates in comparison with MS-H vaccine

Deletions and Insertions

amino acids of the **MSPB** sequence

- There was deletion of **13 amino acids** - NPGTDNSQNPNG - within sequence length of **MSPB** sequence of MS-H, in comparison with the **MSPB** of **studied respiratory isolates**.
- There was deletion of **7 amino acids** - (GNPGTPG - within sequence length of **MSPB** sequence of MS-H, in comparison with the **MSPB** of **studied joint isolates**
- The **joint isolates** had an insertion of 20 amino acids – GNPGTPGNPGTDNSQNPNG - within sequence length when compared with the **MSPB** sequence of the **respiratory isolates**

The melting profile of Ms from respiratory samples

Analysis Selection/Setup				Results
Well	Well Name	Well Type	Ct (dRn)	Tm Product 1 (-R'(T))
A2	25	Unknown	20.87	85.15
B1	3	Unknown	16.99	85.10
B2	26	Unknown	19.84	84.60
D1	11	Unknown	16.33	84.60
E1	12	Unknown	15.91	84.60
G1	14	Unknown	18.95	84.60

The melting profile of Ms from arthritis samples

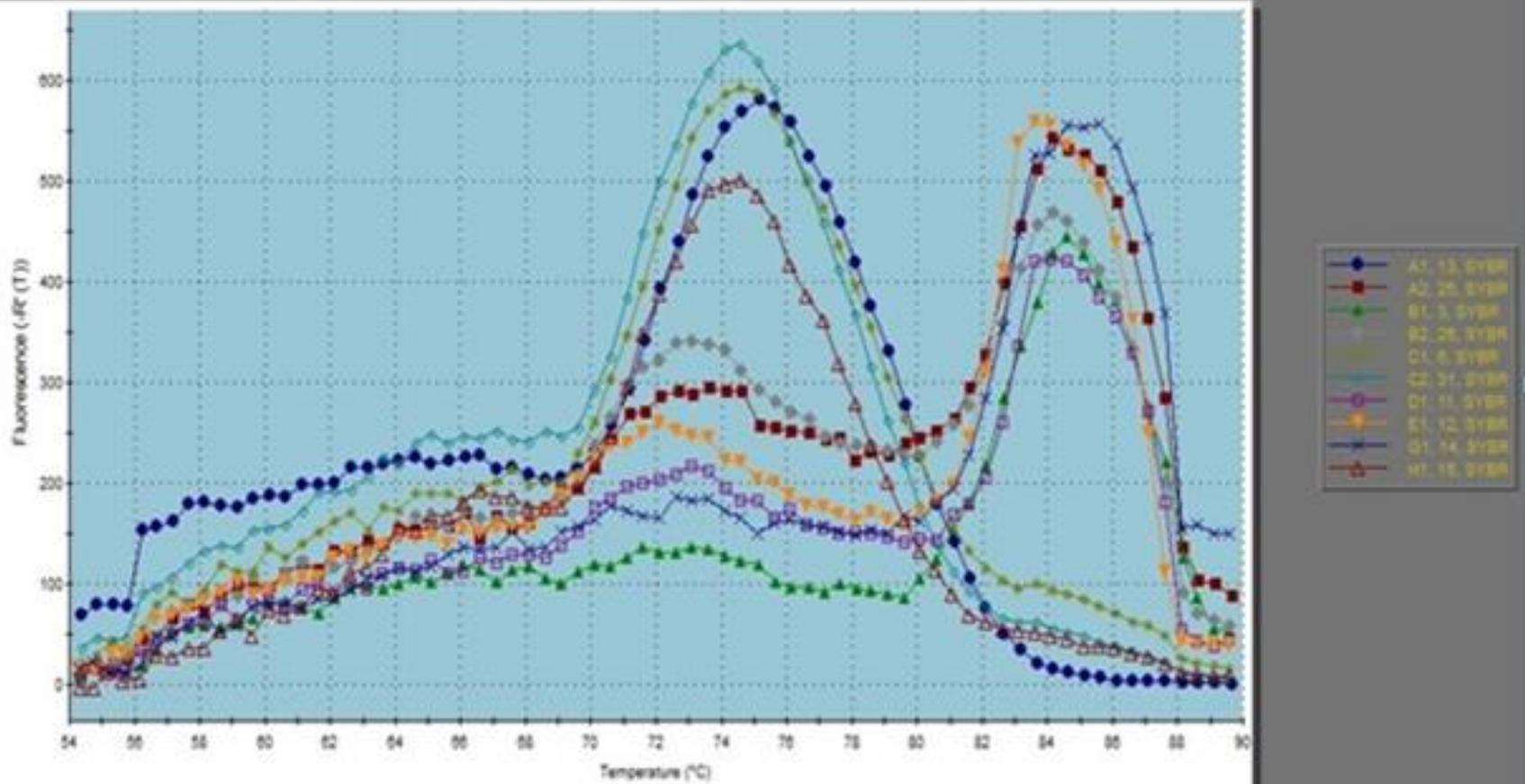
A1	13	Unknown	15.63	74.65
C1	6	Unknown	16.62	74.10
C2	31	Unknown	16.37	74.10
H1	15	Unknown	17.10	73.60

The melting profile of all Ms from arthritis and respiratory samples

Analysis Selection/Setup				Results
Well	Well Name	Well Type	Ct (dRn)	Tm Product 1 (-R'(T))
A2	25	Unknown	20.87	85.15
B1	3	Unknown	16.99	85.10
B2	26	Unknown	19.84	84.60
D1	11	Unknown	16.33	84.60
E1	12	Unknown	15.91	84.60
G1	14	Unknown	18.95	84.60
A1	13	Unknown	15.63	74.65
C1	6	Unknown	16.62	74.10
C2	31	Unknown	16.37	74.10
H1	15	Unknown	17.10	73.60

HRM

Dissociation Curve



HRM

HRM of amplicons using **SYBR green fluorescent dye** of 9 selected **MS** isolates (5 respiratory and 4 arthritic isolates) showed 2 different **HRM** ranges for the isolates:

HRM of **MS** isolates from arthritic joints ranged from **73.6-74.6°C**, while **HRM** for of **MS** isolates from respiratory tissues with inflammatory lesions ranged from **84.6-85.1°C**