Prevalence of *Mycoplasma gallisepticum* in the Ten licensed Hatcheries in Gaza strip, Palestine

by

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A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Master of Biological Sciences/ Medical Technology

2011
Dedication

To the Ministry of Agriculture - Palestine
Acknowledgement

I would like to express my sincere gratitude and appreciation to my supervisors Dr. Abdelraouf Elmanama and Dr. Basim Ayesh, for giving me experience, meticulous valuable advice and support, faithful guidance and close supervision. Thanks are extended to the biological science department staff. I wish to express my deepest gratitude to my family, friends and colleagues.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>CrtmA</td>
<td>Carotenogenic A</td>
</tr>
<tr>
<td>CDC</td>
<td>Center for disease control and prevention</td>
</tr>
<tr>
<td>CEF</td>
<td>Chicken embryo fibroblasts</td>
</tr>
<tr>
<td>CRD</td>
<td>Chronic respiratory disease</td>
</tr>
<tr>
<td>CDS</td>
<td>Coding DNA sequence</td>
</tr>
<tr>
<td>CrmA</td>
<td>Cytokine response modifier A</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>GTS</td>
<td>Gene-targeted sequencing</td>
</tr>
<tr>
<td>GI</td>
<td>Growth inhibition</td>
</tr>
<tr>
<td>HI</td>
<td>Haemagglutination inhibition</td>
</tr>
<tr>
<td>Hela</td>
<td>Human epithelial</td>
</tr>
<tr>
<td>IgA</td>
<td>Immunoglobulin A</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>IgY</td>
<td>Immunoglobulin Y</td>
</tr>
<tr>
<td>IB</td>
<td>Infectious bronchitis</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilobase pairs</td>
</tr>
<tr>
<td>KDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>MAb</td>
<td>Monoclonal antibodies</td>
</tr>
<tr>
<td>MG</td>
<td>Mycoplasma gallisepticum</td>
</tr>
<tr>
<td>Mgcl</td>
<td>Mycoplasma gallisepticum cytadherence membrane 1</td>
</tr>
<tr>
<td>Mgc2</td>
<td>Mycoplasma gallisepticum cytadherence membrane 2</td>
</tr>
<tr>
<td>NPIP</td>
<td>National Poultry Improvement Plan</td>
</tr>
<tr>
<td>ND</td>
<td>Newcastle disease</td>
</tr>
<tr>
<td>OIE</td>
<td>Office International Epizootie</td>
</tr>
<tr>
<td>PNA</td>
<td>Palestinian national authority</td>
</tr>
<tr>
<td>PvpA</td>
<td>Phase-variable putative adhesion protein</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PH</td>
<td>Power of hydrogen</td>
</tr>
<tr>
<td>P52</td>
<td>Proteins 52</td>
</tr>
<tr>
<td>P67</td>
<td>Proteins 67</td>
</tr>
<tr>
<td>PMGA</td>
<td>Proteins of Mycoplasma gallisepticum A</td>
</tr>
<tr>
<td>RAPD</td>
<td>Random amplified polymorphic DNA</td>
</tr>
<tr>
<td>RSA</td>
<td>Rapid slide agglutination</td>
</tr>
<tr>
<td>Real time-PCR</td>
<td>Real time-Polymerase chain reaction</td>
</tr>
<tr>
<td>RRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>Rpm</td>
<td>Rounds per minute</td>
</tr>
<tr>
<td>VlhA</td>
<td>Variable lipoprotein hemagglutinin</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
</tr>
</tbody>
</table>
Abstract

Prevalence of *Mycoplasma gallisepticum* in the ten licensed Hatcheries in Gaza strip, Palestine

Avian mycoplasmas are pathogens of different avian species, especially poultry. MG is the most important mycoplasma. Vertical transmission can occur in eggs laid by naturally infected hens or horizontally within the hatcheries resulting in rapid spread of the disease throughout the flock. It is common in many parts of the world as the primary agent of chronic respiratory disease and sinusitis causing important economic losses in the poultry industry and increase the risk to the overall health of human.

The study was conducted in ten licensed local hatcheries, which hatch more than 16 million of one old day chicks yearly for more than 1200 poultry breeding farms in Gaza strip governorates. The study aimed to determine the prevalence of MG infection in representative samples of 390 non-incubated imported fertilized eggs collected systematically randomly in August 2010 from Gaza strip hatcheries. In addition, the diagnostic value of rapid slide agglutination (RSA) test and real time polymerase chain reaction (real time PCR) technique was compared and the current control procedure was investigated.

The results showed that the prevalence of MG specific antibodies by using RSA test was (36.6%) while the prevalence of MG-DNA by using real time PCR technique was (6.2%). In addition, the imported eggs, which were used for hatching process, were not handled in the best-case specifications and quality control measures were limited or absent. A significant correlation between the eggs weight and the prevalence of MG specific antibodies and between the eggs weight and the prevalence of MG-DNA were found. The poultry industry in Gaza strip is facing the infection of MG in the imported fertilized eggs, and the visual observation and certification assessment are not enough for prevention of MG transmission. The free MG egg sources, bio-safety measures, knowledge and protection programs could be reduce the rate of infection.

**Key words:** *Mycoplasma gallisepticum*, chronic respiratory disease, hatcheries, Gaza strip
ملخص البحث

انتشار جرثومة المايوكوبلازماغالسيبيتكم في العشرة مفرخات المخصبة في قطاع غزة، فلسطين

مايوكوبلازماغالسيبيتكم في الطيور: هي جراثيم مُرضية لجميع أنواع الطيور - خاصة الدواجن -، وتعتبر المايوكوبلازماغالسيبيتكم أكثرها أهمية، وهي تنتقل رائسةً من خلال الأمهات المصابة إلى البيض، وفقًا داخل المفرخات؛ لينتج عن ذلك انتشار سريع للمرض في كل القطاع. إن المايوكوبلازماغالسيبيتكم شائعة في جميع أنحاء العالم، وهي المسبب الرئيسي لمرض التهاب الجهاز التنفسي المزمن، والتهاب الجيوب الأنفية في الطيور، الذي يؤدي إلى خسائر اقتصادية كبيرة جداً في قطاع الدواجن، إضافة إلى الخطر الواقع على الصحة العامة للإنسان.

وقد أجريت الدراسة في العشرة مفرخات مخصحة محليًا في محافظات قطاع غزة، والتي تعمل على إنتاج أكثر من (15 مليون) كتكوت، عمر الوالد منها يومًا، لأكثر من (1200) مزرعة، والتي تعمل على تربية الدواجن في محافظات قطاع غزة; لتحديد انتشار العدوى بجرثومة المايوكوبلازماغالسيبيتكم تم جمع عينة عشوائية مكونة من 390 بيضة مسرودة مخصصة، غير محضنة من مفرخات قطاع غزة في شهر أغسطس 2010 من جانب التحقق من إجراءات الرقابة الحالية، ومقارنة النتائج لكل من اختبار التلازن السريع وتقنية Sلسلة تفاعل البلمرة من جانب آخر.

أظهرت نتائج هذه الدراسة أن نسبة وجود المضادات المندية المخصصة للمايوكوبلازماغالسيبيتكم، باستخدام اختبار التلازن السريع كانت (6.3%)، بينما نسبة وجود الحمض النووي باستخدام تقنية Sسلسلة تفاعل البلمرة كانت بنسبة (6.2%), وأن البيض المستورد المستخدم في عملية الإصابة ليس في أفضل حالاتها، وأن معايير مراقبة الجودة محدودة أو غير موجودة. وأكدت الدراسة على وجود علاقة معنوية بين وزن البيض والمضادات المندية للجرثومة، وبين وزن البيض والحامل النووي لها.

إن صناعة الدواجن في قطاع غزة تواجه الإصابة بجرثومة المايوكوبلازماغالسيبيتكم في البيض المستورد المخصص وإن الطرقية البصرية والشهادة الصحية لبكتريات المنغج العناصر، إن سلامة مصادر البيض، والمعرفة، وإتباع الإجراءات والبرامج الصحية ربما يقلل من معدل الإصابة.

الكلمات المفتاحية: مايوكوبلازماغالسيبيتكم، مرض التهاب الجهاز التنفسي المزمن، المفرخات، قطاع غزة.
Chapter 1
Introduction

1.1 Overview

In Gaza-strip, poultry industry is very important as the consumption of poultry meat and eggs is increasing steadily [1]. The poultry industry is facing various problems. One of these problems is the infection of grandparent and parent flocks, which occurs in many developing countries resulting in dissemination of diseases including mycoplasmosis, salmonellosis and reoviral infection [2].

Mycoplasmas tend to be quite host specific; some infect only a single species of animal while others may have the ability to infect several different animal species. They may be found in humans, many animal species, plants and insects. In general, mycoplasma colonize mucosal surfaces and most species are noninvasive but some species, including *Mycoplasma gallisepticum* (MG) have the ability to penetrate cells and cause diseases [3].

MG is a member of the class Mollicutes, which are very small prokaryotes devoid of cell walls, bounded by a plasma membrane only. This accounts for the (fried egg) type of colony morphology, resistance to antibiotics that affects cell wall synthesis and complex nutritional requirements [4]. It remains the most frequently reported bacterial causative agent of chronic respiratory disease in chickens and infectious sinusitis in turkeys [3].

It is a highly infectious respiratory pathogen affecting poultry. In broilers, MG causes reduction in weight gain, decrease in feed conversion efficiency, increase in mortality rate and increased condemnations in slaughterhouses. In breeders and layers, the disease causes a drop in egg production and an increase in embryo mortality [2]. Inoculation of MG into 7-day-old embryonated chicken eggs via the yolk sac route usually results in embryo deaths within 5-7 days [5].
A survey of commercial egg laying poultry in United States of America (USA) revealed that 37% of laying flocks (262.6 million layers) were infected with MG and causing an annual losses of 97 million US $ [6]. In addition, medication costs make this disease one of the costliest disease problems confronting the poultry industry [7] and causes problems in food safety, drug resistance, and drug residual [2].

MG vertical transmission can occur through eggs or horizontally by inhalation of contaminated airborne droplets, resulting in rapid disease transmission throughout the flock. Prevention and control programs based on strict biosecurity, surveillance, and eradication of infected breeder flocks are preferable. Using vaccination with bacterins has been shown to reduce, but not eliminate, colonization by MG [1].

On some farms, especially in areas with an intensive and varied population of poultry flocks, there may be extensive use of antibiotics for MG [8]. Antibiotics were used to combat the disease, through the application of a single drug administration or drugs in combination, for prevention, control and treatment of MG infection [6]. However because such a solution is not always economically feasible, it is important to be able to detect vertical infection[9].

It is difficult to diagnose MG infections in poultry flocks based on clinical signs. Routine culture procedures and serology are commonly used. The diagnosis of MG infection traditionally has been done by serology [10]. Some of the disadvantages of serological methods are false-positive and false-negative reactions due to interspecies cross-reactions and nonspecific reactions [11]. Recently, office international epizootie and national poultry improvement plan recommended PCR as a reliable test for the detection of MG infections. Real-time PCR, which has distinct advantages over conventional PCR, such as higher reliability, rapidity and prevention of environmental contamination, has been used for the detection of MG in poultry [12].
There is no published data on the prevalence of MG in Gaza strip and to the best of our knowledge, this is the first study investigating the prevalence of this pathogen in fertilized eggs used in the production of one-day-old chicks in hatcheries in Gaza strip.

1.2 Objective

1.2.1 General objective
To determine the prevalence of MG infection in non-incubated imported fertilized eggs in the ten licensed hatcheries of Gaza strip, Palestine.

1.2.2 Specific objectives

● To detect the prevalence of MG antibodies by rapid slide agglutination (RSA) test.
● To detect the prevalence of MG-DNA by real time PCR technique.
● To compare the diagnostic value of RSA test and real time PCR technique.
● To describe the current procedures for control of MG infections.

1.3 Significance

MG economically affects the poultry industry through increased mortality and decreased egg production and reduced feed efficiency. The infected places may undergo serious sequelae and need early and prompt treatment. This study is the first tackling MG, and no previous studies concerning MG were conducted in Gaza strip. There is no available data about MG in the study area and the result of this study may contribute to increase the awareness of the relevant authorities about the prevalence of this pathogen. In addition, data generated from this study may provide the Ministry of Agriculture and other concerned parties a true and scientific view of the existing situation of imported fertilized eggs. This may help in planning and implementing actions to reduce the spread of this costly pathogen.
Chapter 2
Literature Review

2.1 Study area

Gaza strip is a Palestinian administrated territory in the Middle East, boarded on the south by Egypt, on the west by the Mediterranean, and the east and north by "Israel". It is one of the most densely populated places on earth with a total area of 365 km² and population of over 1.54 million [7]. In Gaza strip, agriculture is considered the most important productive sector. More than 20% of the population is dependent upon it. The animal production sector is one of the most important sectors of Palestinian agriculture. Its importance comes from the increasing investments in the livestock sector [13].

This share had increased from 20% to 30% for the years of seventies and nineties, respectively. Gaza strip hatcheries (Table: 2.1) are capable of providing both broiler and layer chickens, they have maximum capacity closed to 70 million of eggs. They are dependent on fertilized eggs, which come from multiple sources, including locally produced as well as imported from "Israel" or from abroad [13]. The quantities of imported fertilized broiler eggs through the first three months of the year 2010 were 9.1 million (Table: 2.2) [14].

Table 2.1: Hatcheries and poultry capacity in Gaza strip [13].

<table>
<thead>
<tr>
<th>Governorate</th>
<th>No. of hatcheries</th>
<th>Capacity eggs/ million</th>
<th>Hatched eggs/ million</th>
<th>No. of Broilers farms</th>
<th>No. of Broilers /million</th>
<th>No. of Layers farms</th>
<th>No. of Layers/ Thousand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gaza north</td>
<td>2</td>
<td>9.4</td>
<td>2.9</td>
<td>108</td>
<td>2.1</td>
<td>26</td>
<td>128</td>
</tr>
<tr>
<td>Gaza</td>
<td>1</td>
<td>19.2</td>
<td>9.4</td>
<td>132</td>
<td>2.2</td>
<td>63</td>
<td>427</td>
</tr>
<tr>
<td>Gaza med</td>
<td>3</td>
<td>18.4</td>
<td>3</td>
<td>230</td>
<td>3.5</td>
<td>26</td>
<td>123</td>
</tr>
<tr>
<td>Khan younis</td>
<td>3</td>
<td>15.6</td>
<td>5.8</td>
<td>353</td>
<td>4.5</td>
<td>21</td>
<td>76</td>
</tr>
<tr>
<td>Rafah</td>
<td>1</td>
<td>7.2</td>
<td>0.4</td>
<td>254</td>
<td>3</td>
<td>6</td>
<td>74.5</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>70</td>
<td>21.6</td>
<td>1077</td>
<td>15.3</td>
<td>142</td>
<td>828.5</td>
</tr>
</tbody>
</table>
Table (2.2): Eggs source and quantities [14].

<table>
<thead>
<tr>
<th>Eggs Source</th>
<th>Hatched eggs / million</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>West Bank</td>
<td>0.2</td>
<td>2.8</td>
</tr>
<tr>
<td>&quot;Israel&quot;</td>
<td>7.6</td>
<td>82.7</td>
</tr>
<tr>
<td>Abroad</td>
<td>1.3</td>
<td>14.5</td>
</tr>
<tr>
<td>Total</td>
<td>9.1</td>
<td>100</td>
</tr>
</tbody>
</table>

2.2 Hatcheries

Hatcheries are an integral link in the chicken supply chain. They come between two producer groups, the broiler hatching egg producers and the broiler producers. Hatchery location is inevitably a compromise between the disease risks of a populated poultry area, the transport costs of eggs and chicks, the availability of labor and the overall transport network [15]. Good hatchery design is essential for cost-effective operation. Their design must therefore incorporate food hygiene standards.

The conditions provided to maintain embryonic growth in the incubators are also ideal for the growth of bacteria and molds. All room surfaces, items of equipment and incubators must be designed to allow simple, regular and effective cleaning and sterilization (Figure 2.1) [15].

Figure (2.1): Al Ghefary hatchery- Gaza strip. Picture was taken by the researcher in 15-8-2010.
2.2.1 Hatchery management

Five major functions are involved in the incubation and hatching of chicken eggs (Table 2.3). The five functions are temperature, humidity, ventilation, egg turning and sanitation. When two or more are not controlled, it may be a disaster. A consistently low temperature will result in a late hatch and decreased hatchability. The chicks may be large, soft bodied and weak. A consistently high temperature will result in an early hatch and decreased hatchability. The chicks may have short down and have rough navels. More chicks will be malformed, deformed legs, weak and small [16].

A sign of low humidity is sticky embryos during pipping and hatching that result in embryos not being able to detach themselves from the shell. In addition, results in short down on the chicks, malformed, mal-positioned, weak and small chicks. As embryos grow, the air vent openings are gradually opened to satisfy increased embryonic oxygen demand. If the egg is not turned, the developing embryo is squeezed between the yolk and shell and can be damaged or killed. Poor sanitation causes not only poor hatch but also subsequent early death during brooding and affects the birds during the grow-out period [16].

Table (2.3): Major functions for incubation and eggs hatching [16].

<table>
<thead>
<tr>
<th>Item</th>
<th>Chicken</th>
<th>Turkey</th>
<th>Duck</th>
<th>Goose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation period (days)</td>
<td>21</td>
<td>28</td>
<td>28</td>
<td>28-34</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>38</td>
<td>37</td>
<td>38</td>
<td>37</td>
</tr>
<tr>
<td>Humidity %</td>
<td>85-87</td>
<td>84-86</td>
<td>85-86</td>
<td>86-88</td>
</tr>
<tr>
<td>No egg turning after</td>
<td>18&lt;sup&gt;th&lt;/sup&gt; d.</td>
<td>25&lt;sup&gt;th&lt;/sup&gt; d.</td>
<td>25&lt;sup&gt;th&lt;/sup&gt; d.</td>
<td>25&lt;sup&gt;th&lt;/sup&gt; d.</td>
</tr>
<tr>
<td>Open additional vents</td>
<td>10&lt;sup&gt;th&lt;/sup&gt; d.</td>
<td>14&lt;sup&gt;th&lt;/sup&gt; d.</td>
<td>12&lt;sup&gt;th&lt;/sup&gt; d.</td>
<td>1&lt;sup&gt;st&lt;/sup&gt; d.</td>
</tr>
<tr>
<td>Open vents (if needed)</td>
<td>18&lt;sup&gt;th&lt;/sup&gt; day</td>
<td>25&lt;sup&gt;th&lt;/sup&gt; d.</td>
<td>25&lt;sup&gt;th&lt;/sup&gt; d.</td>
<td>25&lt;sup&gt;th&lt;/sup&gt; d.</td>
</tr>
</tbody>
</table>

2.3 Chicken egg

2.3.1 Egg weigh

All chicken hens start egg production laying small and gradually increase to a mature egg grade size. Average egg weight increased from 54 gm at 26
weeks of age to 64 gm at 66 weeks of age [17]. Eggs are categorized according to weigh as large, medium, small (Table: 2.4).

Table (2.4): Egg weight categories [18].

<table>
<thead>
<tr>
<th>Size</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large</td>
<td>63 – 73 gm</td>
</tr>
<tr>
<td>Medium</td>
<td>53 – 63 gm</td>
</tr>
<tr>
<td>Small</td>
<td>Less than 53 gm</td>
</tr>
</tbody>
</table>

2.3.2 Egg structure and characteristics

Albumin is a clear liquid formed from the layers of secretions of the anterior section of the hen's oviduct during the passage of the egg. It protects the egg yolk and provides additional nutrition for the growth of the embryo. An egg yolk feeds the developing embryo. It is suspended in the egg white by one or two spiral bands of tissue called the chalazae. The yolk is enclosed by a thin vitelline transparent membrane (Figure 2.2) [19].

Figure (2.2): Egg structure. (a) Sagittal section through a hen's egg, (b) enlargement of the region of the shell shown in (a) [19].
The whole egg composition percentage is 65.5% water, 11.8% protein, 11.0% fat, 11.7% ash. The albumen and yolk physicochemical properties are illustrated in table 2.5 [18].

**Table (2.5):** Physicochemical constants of yolk and albumen adapted from [18].

<table>
<thead>
<tr>
<th>Property / factor</th>
<th>Albumen</th>
<th>Yolk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bound water (%)</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>Coagulating temperature(C)</td>
<td>61</td>
<td>65</td>
</tr>
<tr>
<td>Density (gm/cm3)</td>
<td>1.035</td>
<td>1.035</td>
</tr>
<tr>
<td>Electrical conductance (mho-cm-1 x 10-3)</td>
<td>8.68</td>
<td>3.1</td>
</tr>
<tr>
<td>Freezing point</td>
<td>-0.424</td>
<td>-0.587</td>
</tr>
<tr>
<td>Heat of combustion (cal/gm)</td>
<td>5,690</td>
<td>8,124</td>
</tr>
<tr>
<td>pH</td>
<td>7.6</td>
<td>6</td>
</tr>
<tr>
<td>Refractive index</td>
<td>1.3562</td>
<td>1.4185</td>
</tr>
<tr>
<td>Solubility coefficient for CO2</td>
<td>0.71</td>
<td>1.25</td>
</tr>
<tr>
<td>Specific heat (cal/gmC)</td>
<td>0.85</td>
<td>0.78</td>
</tr>
<tr>
<td>Specific resistance (ohm-cm)</td>
<td>0.12</td>
<td>0.32</td>
</tr>
<tr>
<td>Surface tension (dyn/cm)</td>
<td>53</td>
<td>35</td>
</tr>
<tr>
<td>Vapor pressure (in % of NaCl)</td>
<td>0.756</td>
<td>0.971</td>
</tr>
<tr>
<td>Viscosity (poise at 0C)</td>
<td>25</td>
<td>200</td>
</tr>
<tr>
<td>Latent heat (But/lb)</td>
<td>127</td>
<td>81</td>
</tr>
</tbody>
</table>

**2.3.3 Maternal antibodies**

Chickens transmit maternal antibodies to their offspring by depositing the antibodies in the egg. There are three classes of antibodies in chickens, namely IgY (IgG), IgA, and IgM. Chicken IgA and IgM are similar to mammalian IgA and IgM in terms of molecular weight, structure, and immunoelectrophoretic mobility. Although structural differences exist between IgY and mammalian IgG, IgY is considered the avian equivalent to mammalian IgG. In eggs, IgY is present in the egg yolk, whereas IgA and IgM are present in the albumen as a result of mucosal secretion in the oviduct. There is transfer of IgA and IgM antibodies from the albumen into the egg yolk [20].
2.4 *Mycolplasma*

2.4.1 History

The first accurate description of the avian mycoplasmosis was in 1905 by Dodd in England and termed Epizootic pneumoenteritis [21]. In 1938, Dickinson and Hinshaw named the disease (infectious sinusitis) of turkeys [22]. In 1943, Delaplane and Stuart cultivated an agent in embryos isolated from chickens with chronic respiratory disease (CRD) and later from turkeys with sinusitis [23]. In the early 1950, Markham, Wong, and Van reported that the organism was a member of the Pleuropneumonia group [24].

2.4.2 MG organism

MG (Figure 2.3) is an avian pathogen within the genus mycoplasma (class Mollicutes) which includes other species infecting animals, humans, insects and plants [25]. The organism stains well with Giemsa stain, but weakly gram negative. It is generally coccoid, approximately (0.25-0.5 μm) [4]. The organism shows a filamentous or flask-shaped polarity of the cell body due to the presence of terminal organelles or bleb [26]. MG requires a protein-rich medium for their growth, containing 10-15% added animal serum [27].

![Figure (2.3): A hypothetical MG scheme cell. (a) The specialized terminal structure, (b) the electron-dense area (infrableb), (c) loop-shaped tubules and (d) plasma membrane [28].](image-url)
2.4.3 Taxonomy of MG

The Mollicutes (mollis=soft and cutes = skin), belong to the phylum Firmicutes with low Guanine + Cytosine content of the genome, belongs to the domain bacteria. About 200 species of the class Mollicutes have been validly described [29]. It was first classified and differentiated from other avian mycoplasmas by serotyping [30] and was commonly designated serotype A [31]. Mycoplasma phylogeny and taxonomy continue to be reexamined by the application of molecular tools such as DNA sequence analysis [25]. Taxonomy of MG is shown in table 2.6.

Table (2.6): Taxonomy of MG [32].

<table>
<thead>
<tr>
<th>Taxonomy ID</th>
<th>233150</th>
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</thead>
<tbody>
<tr>
<td>Kingdom</td>
<td>Bacteria</td>
</tr>
<tr>
<td>Intermediate Rank 1</td>
<td>Firmicutes</td>
</tr>
<tr>
<td>Intermediate Rank 2</td>
<td>Mollicutes</td>
</tr>
<tr>
<td>Intermediate Rank 3</td>
<td>Mycoplasmataceae</td>
</tr>
<tr>
<td>Intermediate Rank 4</td>
<td>Mycoplasma</td>
</tr>
<tr>
<td>Intermediate Rank 5</td>
<td><em>Mycoplasma gallisepticum</em></td>
</tr>
</tbody>
</table>

2.4.4 MG structure and characteristics

The Mollicutes have only the plasma membrane (Figure 2.4) which proteins constitute over two-thirds of the mass, with the rest being membrane lipids. Motif analysis of the MG genome has predicted a large repertoire of membrane-associated proteins. Of these proteins, 149 contain multiple transmembrane domains and 24 ATP-binding proteins predicted to be involved in transport of biomolecules [33].

The membrane lipoproteins are a majority of the mycoplasma surface antigens [34]. All mycoplasma lipids are located in the cell membrane and consist of phospholipids, glycolipids and neutral lipids. The mycoplasmas are partially or very incapable of fatty acid synthesis and require cholesterol for growth [35].
The genome is composed of 996,4 bp with an overall Guanine + Cytosine content of 31%. It contains 742 putative coding DNA sequences (CDSs), representing a 91% coding density. Function has been assigned to 469 of the CDSs, while 150 are conserved hypothetical proteins and 123 remain as hypothetical proteins [37]. MG genome contains two copies of the rRNA genes. One set is organized as an operon, with adjacent 16S, 23S and 5S genes, and a second copy of the 16S rRNA gene lies 221 kb upstream of the 23S and 5S rRNA genes [33].

The organization of the putative origin of replication for MG is located in the region of the dnaA gene (Figure 2.5) [33]. It has been well established that MG generally expresses a single member of the family at any one time [35]. The specific gene expressed can be influenced by growth in the presence of cognate antibody [36]. The probable role of this family in generating antigenic variation has been demonstrated in infected chickens [7].

*M. gallisepticum*

![Diagram of Mycoplasma cell membrane and origin of replication](image)

**Figure (2.4):** Mycoplasma cell membrane [36].

**Figure (2.5):** Origin of replication of MG [33].
2.4.4.1 Antigenic structure and toxins

The plasma membrane of MG contains approximately 200 polypeptides associated with surface antigenic variation, adhesion to host cells, motility and nutrient transport [2]. Considerable effort has been made to identify MG adhesion or hemagglutinin properties, which may play key roles in the pathogenesis of an immune response to infection. Adhesins are integral membrane proteins having regions exposed on the cell surface that attach to receptor sites on host epithelial cells, which allow for colonization and infection. Such as these proteins considered important virulence factors and antigens [38].

Immunoblotting techniques revealed that the surface antigens p52 and p67 (pMGA), were specific to MG and the closely related species. The pMGA gene family is significant genomic commitments to antigenic variability and hypothesized function of immune evasion [39]. The pMGA gene family also provides a mechanism for rapid and reversible switches in its expression of proteins (antigenic switching) in response to antibodies or other environmental cues [37]. In 2003, the pMGA gene and protein were renamed vlhA and VlhA respectively [33].

The vlhA gene family encodes hemagglutinin in MG, and the vlhA genes are located in several loci around the chromosome and antigenic variation is generated by alternating transcription of over 40 translationally competent genes [40]. PvpA is an MG size-variable integral membrane protein that shows high-frequency phase variation in its expression and adds to the complexity of antigenic variation in MG. Antigenic variation and expression of PvpA and p67a (major immunogenic surface proteins) were correlated with antibody response in vivo, suggesting that immune modulation may have a key role in generating surface diversity [41].

The preceding information and that from many other reports indicates that the MG genome is highly committed to antigenic variation and variable
expression of surface proteins [42]. Other adhesion proteins identified in MG are GapA and Mgc2. GapA is a primary cytadhesin that appears to work in a coordinated way with at least one other cytadherence-related protein, CrtnA undergoing concomitant phase variation in expression [43]. Expression of these two components has been correlated with binding to erythrocytes and efficient attachment to cultured cells. These results demonstrated that both GapA and CrmA are required for MG cytadherence and pathogenesis. Potent toxins have not been associated with mycoplasmas [7].

2.4.5 Epidemiology
MG has been isolated from naturally occurring infections in chickens (Gallus gallus), turkeys (Meleagris gallopavo), pheasants (Phasianus colchicus) chukar partridge (Alectoris graeca cristatus) [44], bobwhite quail (Colinus virginianus) and Japanese quail (Coturnix japonica) [45]. MG has also been isolated from duck (Anas platyrhynchos), from geese (Anser anser), from house finches (Carpodacus mexicanus) [46], from a golden pheasant (Chrysolophus pictus) in Australia [47], from a yellow-naped Amazon parrot (Amazona ochrocephala auropalliata) [48].

MG infection has been produced experimentally in captive - reared wild turkeys [49], House sparrows (Passer domesticus) and budgerigars (Melopsittacus undualtus) [50]. MG was also isolated from a blue jay (Cyanocitta cristata) that contracted conjunctivitis and from free-ranging American goldfinches (Carduelis tristis) [51]. MG have been confirmed by culture or PCR in purple finches (Carpodacus purpureus), eastern tufted titmice (Baeolophus bicolor), pine grosbeaks (Pinicola enucleator) and evening grosbeaks (Coccothraustes vespertinus) [52].

MG causes disease in game birds including pheasants (Phasianus colchicus), chukar partridges (Alectoris chukar), bobwhite quail (Colinus virginianus), Japanese quail (Coturnix japonica) and peafowl (Pavo cristatus), pigeons, greater flamingos (Phoenicopterus roseus), wild peregrine falcons (Falco
peregrines) in Spain [52] MG has the ability to invade cultural human epithelial cells (Hela -229) and chicken embryo fibroblasts (CEF) (Figure 2.6) and survive within the intracellular space over a 48-h period [53].

![MG interaction with CEF cells](image)

**Figure (2.6):** MG interaction with CEF cells, localization of extracellular (yellow) and intracellular (red). Bars, 10 mm [53].

### 2.4.6 Morbidity and mortality

MG is not a killer disease like Newcastle (ND) or Gumboro but, in complicated cases, birds may die [54]. Inoculation of broth cultures or exudates containing MG into 7-day-old embryonated chicken eggs via the yolk sac route usually results in embryo deaths within 5-7 days. The organism reaches its highest concentration in the yolk sac, yolk and chorioallantoic membrane just prior to embryo death. MG embryo mortality was prevented in eggs containing maternal MG antibodies [55].

MG infection usually affects nearly all chickens in a flock but disease is variable in severity and duration. It tends to be more severe and of longer duration in the cold months and affects younger birds, more severely than mature birds [56]. Mortality may be negligible in adult laying flocks, but there can be a reduction in egg production [57]. In broilers, the mortality may range from low in uncomplicated disease to as much as 30% in complicated outbreaks, especially during the colder months. Retarded growth, downgrading of carcasses and condemnations constitute further losses [2].
2.4.7 Transmission
Horizontal transmission occurs by direct or indirect contact of susceptible birds with infected clinical or subclinical birds resulting in high infection disease prevalence within flocks. The upper respiratory tract and or conjunctiva are portals of entry for the organism in aerosols or droplets [2]. MG survived in the human nasal passage for 24 hours; on straw, cotton, and rubber for 2 days; on human hair for 3 days; and on feathers for 2-4 days [58]. MG seldom survives for more than a few days outside of a host, so clinical or subclinical carrier birds are essential to the epidemiology of MG diseases [59]. However, outbreaks may occur via fomites; contaminated airborne dust, droplets or feathers, coupled with suboptimal biosecurity and personnel practices [59].

MG vertical transmission (in ovo transovarian) is known to occur in eggs laid by naturally infected chicken hens [2]. The highest rates of transmission were found during the acute phase of the disease when MG levels in the respiratory tract peaked [60]. In separate studies, peak egg transmission was detected four weeks after MG challenge in approximately 25% of the eggs and at three to six weeks post challenge in more than 50% of the eggs. On a flock basis, egg transmission rates decline as the post infection interval lengthens. Transmission rates of approximately 3% at 8-15 weeks [61] and approximately 5% at 20-25 weeks have been reported. During chronic infections under field conditions, egg transmission is likely to occur at much lower levels [2].

2.4.8 Incubation period
In experimental infections of chickens, the MG incubation period varies from 6-21 days depending on MG strain virulence, complicating infections (polymicrobial infections), and environmental and other stressors [2]. Therefore, under natural conditions it is very difficult to estimate the possible date of exposure based on the appearance of clinical signs. Many variable factors seem to influence the onset and extent of clinical disease [62].
Chickens often develop clinical infections near the onset of egg production. This apparent extended incubation period was especially common in offspring of infected chicken hens hatched from eggs dipped in antibiotic solutions for control of MG infection. The possible role of contamination from other sources of infection is not always clear and can rarely be proved beyond reasonable doubt [62].

2.4.9 Clinical signs
The clinical manifestations of MG in chicken embryo are dwarfing, generalized edema, liver necrosis and enlarged spleens [55]. In other side, the clinical signs in chicken include coughing, sneezing, snicks, rales, ocular and nasal discharge, decrease in feed consumption and egg production, increased mortality, poor hatchability and lose weight [63]. The gross lesions in birds with MG include catarrhal inflammation of sinuses, trachea and bronchi. Air sacs are often thickened and opaque, and may contain mucous or caseous exudates, besides hyperplastic lymphoid follicles on the walls [63].

At slaughter, carcass condemnation may result from the presence of airsacculitis (Figure 2.7), fibrinous perihepatitis and adhesive pericarditis; interstitial pneumonia and salpingitis, which are often seen in chickens [63].

Figure (2.7): Severe airsacculitis with abundant foam and aggregates of caseous exudates associated with MG infection [64].
Chickens conjunctivitis, keratoconjunctivitis caused by MG was reported in commercial layer chickens [65]. Chickens showed swelling of the facial skin and the eyelids, increased lacrimation, congestion of conjunctival vessels. However, flocks may have serologic evidence of infection with no obvious clinical signs, especially if they encountered the infection at a younger age and have partially recovered. Male birds frequently have the most pronounced signs and the disease is often more severe during winter [66].

2.4.10 Economic impact
MG is the most pathogenic and economically significant mycoplasma pathogen of poultry [67]. MG economically affects the poultry industry through increased mortality and decreased egg production [68] and reduced feed efficiency [69]. Some researchers demonstrated that the average egg production loss due to naturally exposed MG infection, which was 15.7 eggs per chicken hen as compared with MG -free hens [68]. Other researchers showed that MG infected flocks produced 5-12 fewer eggs per chicken hen compared with uninfected flocks [51].

A statistically significant reduction in egg production was observed during the first 4 weeks post exposure to MG and significantly lower fertility of eggs [70]. Airsacculitis in chickens can cause significant condemnations at slaughter. Increased medication costs are additional factors that make this disease one of the costliest disease problems for poultry industry [69]. In the late 70's in USA, MG was costing the poultry industry and consumer as much as 118 million US $ per year, not including losses affecting the broiler industry or other losses in the layer industry such as morbidity, mortality or feed conversion [71].

Documented turkey industry losses in north Carolina, USA, due to MG between 1979 and 1983 were greater than 2.5 million US $ [72]. The commercial layer producers in southern California lost an estimated 127 million eggs because of MG in 1984. This lost egg production and associated
MG control program costs amounted to an estimated financial loss of approximately 7 million US $. This represented a loss of approximately 6 million US $ in consumer surplus [57]. In Brazil, there was a loss of 34 thousand tons of broilers in the end of the production cycle due to respiratory diseases, which corresponded to 30 million US $ in 1994. Moreover, MG infection alone is considered one of the diseases that cause more losses to the poultry industry [68]. Prevention and control programmes of MG includes chemotherapy, vaccination etc, are account for additional costs [6].

2.5 Diagnostic techniques
The presence of MG can be confirmed by isolating the organism in a cell-free medium or by detecting its DNA directly in infected samples. Serological tests are also widely used for diagnosis [73]. Samples are taken from live and dead birds [74]. Several suitable culture media have been formulated. MG media generally contain a protein digest and a meat-infusion base supplemented with serum or a serum fraction, yeast factors, glucose and bacterial inhibitors [75].

The most commonly serological tests used is rapid slide agglutination test (RSA). It should be carried out at room temperature (20-25° C) within 72 hours of sample collection [75]. The positive results should be considered presumptive for the presence of MG antibodies [76]. MG in haemagglutination inhibition test is capable of haemagglutinating avian red blood cells, and specific antibodies in test sample cause inhibition. A strain should be selected that grows well and haemagglutinates reliably [77].

In Enzyme-linked immunosorbent assay the plates are coated with whole cell MG antigen and the test samples are added, but the reaction is assessed by the extent of blocking that occurs when the conjugated monoclonal antibody is added [78]. In growth inhibition test, the growth of MG is inhibited by specific antiserum, enabling species to be identified. It is relatively insensitive and sera must be high- titred, monospecific [74].
MG may be detected by hybridization with DNA probes, but now it is much more common to use the PCR to amplify specific portions of DNA in the test material. Molecular methods are also available for differentiation of MG strains [78]. DNA fingerprinting uses arbitrary primed PCR or random amplified polymorphic DNA (RAPD). Gene-targeted sequencing (GTS) for the mgc2, gapA, pvpA, and MG A_0309 genes can be used to provide an accurate and reproducible method of typing of strains [79].

2.5.1 Differential diagnosis
To differentiate MG infection from other common respiratory diseases. Newcastle disease (ND) and Infectious bronchitis (IB) diseases or their antibodies may be present as separate entities or as part of the complicated chronic respiratory disease (CRD) syndrome. Infectious coryza (Avibacterium paragallinarum) and fowl cholera (Pasteurella multocida) usually can be identified by bacterial culture. Mycoplasma synoviae infection may be present alone or in addition to MG. Application of both serologic and organism identification test procedures may be necessary in some cases [66].

2.6 Prevention and control
The control strategy of many countries is based on maintaining MG free breeding flocks. Establishing the MG - clean status of breeder flocks and maintaining that status can be accomplished by participation in control programmes [80].

2.6.1. Immunity and mucosa immune system
Chickens that have recovered from clinical signs of MG diseases are known to have some degree of immunity. However, recovered birds may still carry the organism [81] Antibodies persisted in recovered chickens, and upon reexposure, they had a faster rate of MG elimination and less severe tracheal lesions than observed after the first exposure. The importance of antibodies produced in response to MG infection inhibited attachment of the organism to epithelial cells [82].
Mycoplasmas may affect the cell-mediated immune system by inducing either suppression or stimulation of B and T lymphocytes, and inducing cytokines. Lymphoproliferation, interferon and nitric oxide were detected in vitro in antigen-stimulated peripheral blood leukocytes from MG-infected chickens [83]. The primary role for local antibody mediated responses in controlling MG infection, but also presented evidence for significant natural killer and cytotoxic T cell responses to infection [84].

2.6.2 Vaccination
Vaccination is the preferred method for control and maintains MG-free flocks. It should be considered only in situations where field exposure is inevitable, such as on multi-age sites and potential exposure of neighboring poultry flocks. Successfully MG vaccinated birds are resistant to respiratory disease, airsacculitis, egg production drops and reduced levels of egg transmission in breeders [67]. Two types of vaccines are available for the control of MG [85]. Vaccinated chickens with MG F, MG Ts-11 Live vaccines strains (intranasal or eye drop) and MG 6/85 strain (as a fine spray) are permanent carriers, so a single dose is adequate [86].

Use of MG F strain vaccine in each replacement flock on a multi-age site will eventually result in displacement of the field strain with the vaccine strain. MG Ts-11 and 6/85 strains are a virulent and spread to unvaccinated birds does not occur or occurs very poorly when birds are in very close contact [86]. MG Inactivated vaccines (bacterins) are prepared from a concentrated suspension of whole cells that is emulsified into an oil adjuvant. High antigen content is essential [87]. Hens vaccinated with live MG strain F and one dose, or two doses of bacterin, was significantly lower level than unvaccinated controls [60].

2.6.3 Medication
MG has shown sensitivity in vitro and in vivo to several antimicrobics including macrolides, tetracyclines, fluoroquinolones and others however
resistance to penicillins and other antibiotics, which act by inhibiting cell wall biosynthesis occurs. MG may develop resistance, and demonstrate cross-resistance, to commonly used antibiotics [88]. Antimicrobics have been used to treat MG to reduce egg production losses, may reduce the severity of clinical signs and lesions and significantly reduce populations of MG in the respiratory tract [89,90,91].

Egg injection or dipping has been used to introduce antimicrobials into hatching eggs to control MG in ovo transmission [92]. In general, these methods greatly reduced, but sometimes did not completely eliminate, the possibility of egg transmission. However, the use of antimicrobials for egg injection or dipping made it possible to obtain sufficient MG free birds [93].

2.6.4 Hazards of residual drugs in poultry products

Use of antibiotic that might result in deposition of residues in meat and eggs must not be permitted in food intended for human consumption. If use of antibiotics is necessary as in prevention and treatment of animal diseases, a withholding period must be observed until the residues are negligible or no longer detected [94]. The withdrawal period is the necessary interval between the last administration of the drug under normal conditions of use and the time when treated animals can be slaughtered for the production of safe foodstuffs. The withdrawal period should provide a high degree of assurance both to the producers and the consumers that the concentration of residues in foods derived from treated animals are not above the maximum residue levels [95].
# Chapter 3

## Materials and Methods

### 3.1 Materials

#### 3.1.1 Equipments

<table>
<thead>
<tr>
<th>#</th>
<th>Item</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Digital balance</td>
<td>ACCULAB-CANADA</td>
</tr>
<tr>
<td>2</td>
<td>Digital dry bath incubator</td>
<td>BIO TAD- GERMANY</td>
</tr>
<tr>
<td>3</td>
<td>Freezer, refrigerator</td>
<td>ORSO, PHARMAL-SPAIN</td>
</tr>
<tr>
<td>4</td>
<td>Autoclave</td>
<td>CRISTOFOLI-BRAZIL</td>
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<tr>
<td>5</td>
<td>Vortex mixer</td>
<td>TURBO-USA</td>
</tr>
<tr>
<td>6</td>
<td>PCR work station with UV light</td>
<td>BIOTEK</td>
</tr>
<tr>
<td>7</td>
<td>Step one Real time PCR system</td>
<td>APPLIED BIOSYSTEMS</td>
</tr>
<tr>
<td>8</td>
<td>Mini centrifuge</td>
<td>TOMOS</td>
</tr>
</tbody>
</table>
| 9  | Quality micropipettes  
  **• 0.5-10 µl**  
  **• 5.0-50 µl**  
  **• 50-200 µl**  
  **• 100-1000 µl** | JENCONS                  |

#### 3.1.2 Kits, reagents and disposables

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<thead>
<tr>
<th>#</th>
<th>Item</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>SPAFAS MG plate antigen</td>
<td>CHARLES RIVER-USA</td>
</tr>
<tr>
<td>2</td>
<td>QIAamp MinElute DNA kit</td>
<td>QIAGEN-USA</td>
</tr>
<tr>
<td>3</td>
<td>MYCO G/S real time kit</td>
<td>BIONOTE-KOREA</td>
</tr>
<tr>
<td>4</td>
<td>Ethanol (96–100%)</td>
<td>SNOW-USA</td>
</tr>
<tr>
<td>5</td>
<td>Phosphate buffer saline</td>
<td>SIGMA-GERMANY</td>
</tr>
<tr>
<td>6</td>
<td>Disposable sterile plastic pipette</td>
<td>LABCON-USA</td>
</tr>
<tr>
<td>7</td>
<td>Sterile specimen collection vials</td>
<td>LABCON-USA</td>
</tr>
</tbody>
</table>
| 8  | Filter tips  
  **• 0.5-10 µl**  
  **• 10-200 µl**  
  **• 100-1000 µl** | LABCON-USA                |
| 9  | 1.5, 2 ml Microcentrifuge tubes                    | LABCON-USA                |
3.2 Methods

3.2.1 Sampling

3.2.1.1 Sample size calculation
Because the proportion of MG is unknown since there is no previous studies carried out in this subject, we considered $P = 0.050$, with 95% confidence interval and the maximum error of estimate = 0.05. The estimated representative sample size was 390 samples [96].

3.2.1.2 Study population
The current study was cross-sectional descriptive study and was conducted according to good clinical practice guidelines recommended by the office international epizootie (OIE). The study included eggs collected from ten local licensed hatcheries in Gaza strip and imported either from Israel or West Bank.

3.2.1.3 Samples collection
Eggs were collected during August 2010 after the shipments arrived from the border checkpoint to the egg room storage in each hatchery. A specialized veterinary doctor (Ministry of agriculture) collected the samples systemically, randomly and individually from different shipments and sources.

3.2.1.4 Sample transport and storage
Eggs were transported under appropriate conditions to protect their identity, integrity and biosecurity to the Islamic University-Gaza laboratory on the day of collection then stored in the refrigerator at (4-6°C) prior to processing.

3.2.1.5 Samples processing
The source of eggs, date, and weight were recorded then the egg washed with soap, water and 70% alcohol for surface disinfection. Eggs were broken in individual containers. For RSA test, Albumen were transferred to collection tubes, vortexed and diluted in two-fold dilution by sterile 1 X PBS to minimize the risk of false positive reactions then stored in refrigerator at (4-6 °C) [97]. The samples were tested within 72 hours. For real time PCR
technique, Eggs vetilline membrane with attached yolk was placed into 1.5 ml microcentrifuge tubes containing 700 µl of sterile 1 X PBS then vortexed and stored at -20 °C (Figure 3.1).

Figure (3.1): Samples processing. (a) The egg after cleaned and disinfecting, (b) broken egg, (c) transfer of vetilline samples, (d) storage of the samples. Picture was taken by the researcher in 03-8-2010.

3.2.2 Rapid slide agglutination (RSA) test

3.2.2.1 Test principle
RSA test was used to detect specific antibodies that will bind to an antigen and cause visible "clumping" or agglutination. The antigen should be stored in the dark at (2-7 °C). A prescribed amount of antigen is placed on a solid support, such as a glass plate or mirror, keeping each drop of antigen separate. An equal amount of test sample is placed next to the antigen and these are then mixed together. After a short incubation, the mixture is examined for evidence of agglutination, which appears as discrete clumps of the stained particles with a clearer background. If no antibodies are detected, the mixture will remain opaque.

3.2.2.2 Test procedure
The RSA test The test was performed according to the kit instructions (Carles river-USA). All components (solid plate, antigen, controls, and test sample)
were allowed to warm to room temperature before use. Known positive and a known negative control samples were tested at the start of all testing sessions. Separate pipettes were used for each control. One drop of positive control was placed in one square and one drop of negative control in another square. One drop of each test sample was placed into separate squares. Disposable tips were used between samples, the antigen was shaken well to mix, one drop of the antigen was placed onto each square on the solid plate, the sample was mixed with the antigen, and each mixture was kept within one square. The timer was started set for 2 minutes and the plate was gently rotated for a few seconds, then let stand.

After one minute, the plate was rotated again, allowed to stand, and the reactions were recorded when the 2 minutes reached. Formation of discrete clumps of stained material normally was visible in positive reaction. Negative reactions showed little change in the opaque mixture after 2 minutes (Figure 3.2).

![Figure (3.2): Rapid slide agglutination test.](image)

(a) Positive agglutination, (b) suspect and (c) negative agglutination. Picture was taken by the researcher in 05-8-2010.

### 3.2.3 Real time polymerase chain reaction (real time PCR) technique

#### 3.2.3.1 Pooling of samples

In the pooling strategy, ten samples were pooled together in one tube. Two hundred µl from each sample were combined together in a single 2 ml
microcentrifuge. The tubes were centrifuged at low speed for one minute and the supernatant was transferred to new 2 ml microcentrifuge. The tubes were ultra centrifuged for three hours at 14000 rpm. A pellet was visible and the supernatant was reduced to approximately 250 μl by removal of most of the liquid. The pellet was resuspended in the remaining supernatant and DNA was extracted for real time PCR amplification. The samples of a positive pool were reanalyzed individually. In order to make sure that the detection limit of real time PCR is not compromised, one previously known MG positive sample was introduced separately into a pool of another 9 negative samples as described above.

DNA was extracted from the pool and analyzed by real time PCR for MG. DNA was also extracted from the original non-pooled samples and analyzed by real time PCR in parallel to assess detection capacity of the procedure. DNA extraction from each RSA positive sample was processed individually for real time PCR while negative and suspect RSA samples were extracted and processed in pools of ten samples and when the pool gave positive real time PCR result, each sample in the pool was processed apart.

3.2.3.2 DNA extraction procedure

DNA extraction was performed according to kit instructions (Qiagen-USA). The 25 μl QIAGEN Protease, 200 μl of sample and 200 μl buffer AL (containing 28 μg/ml of carrier RNA) were mixed into a 1.5 ml microcentrifuge tube. The cap was closed and mixed by pulse-vortexing for 15 s and incubated at 56 °C for 15 min in a heating block. The 1.5 ml tube was briefly centrifuged to remove drops from the inside of the lid and 250 μl of ethanol (96-100%) were added to the sample, the cap was closed and mixed thoroughly by pulse-vortexing for 15 s.

The lysate was incubated with ethanol for 5 min at room temperature (15-25 °C). The 1.5 ml tube was briefly centrifuged to remove drops from the inside of the lid and all of the lysate was carefully applied onto the QIAamp MinElute
column. The cap was closed and centrifuged at 8000 rpm for 1 min. The QIAamp MinElute column was placed in a clean 2 ml collection tube, and the collection tube containing the filtrate was discarded. Five hundred μl of Buffer AW1 were added to the QIAamp MinElute column and centrifuged at 8000 rpm for 1 min. The QIAamp MinElute column was placed in a clean 2 ml collection tube, the collection tube containing the filtrate was discarded. Five hundred μl of buffer AW2 were added to the QIAamp MinElute column and centrifuged at 8000 rpm for 1 min.

The QIAamp MinElute column was placed in a clean 2 ml collection tube, the collection tube containing the filtrate was discarded. Five hundred μl of ethanol (96–100%) were added to the QIAamp MinElute column and centrifuged at 8000 rpm for 1 min. The QIAamp MinElute column was placed in a clean 2 ml collection tube and centrifuged at full speed 14000 rpm for 3 min to dry the membrane completely. The QIAamp MinElute column was placed in a clean 2 ml collection tube and the lid was opened and incubated at 56 °C for 3 min to dry the membrane completely to evaporate any remaining liquid.

The QIAamp MinElute column was placed in a clean 1.5 ml microcentrifuge tube, the collection tube with the filtrate was discarded, and 20-150 μl of buffer AVE or RNase-free water were applied to the center of the membrane, incubated at room temperature for 1 min and centrifuged at full speed 14000 rpm for 1 min.

3.2.3.3 DNA amplification and interpretation
Template DNA was amplified using the MYCO G/S real time kit according to the kit instruction. To detect a potential contamination, a positive and negative control was run every time the kit was used. A master mix and real time PCR reaction was prepared as described in tables 3.1 The qualitative assay interpretation of the results were classed based on the criteria of the test as in table 3.2.
Table (3.1): Tube components for DNA amplification

<table>
<thead>
<tr>
<th>#</th>
<th>Reagents</th>
<th>Volume per reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>MG Detection Solution</td>
<td>3.68 µl</td>
</tr>
<tr>
<td>2.</td>
<td>2 X enzyme buffer</td>
<td>8 µl</td>
</tr>
<tr>
<td>3.</td>
<td>Enzyme mix</td>
<td>0.32 µl</td>
</tr>
<tr>
<td>4.</td>
<td>Rox reference dye</td>
<td>0.32 µl</td>
</tr>
<tr>
<td></td>
<td><strong>Total volume</strong></td>
<td><strong>12.3 µl</strong></td>
</tr>
</tbody>
</table>

Table (3.2): Qualitative assay interpretation

<table>
<thead>
<tr>
<th>#</th>
<th>Ct value</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Negative control ≤</td>
<td>Negative</td>
</tr>
<tr>
<td>2.</td>
<td>Negative control &gt;</td>
<td>Positive</td>
</tr>
</tbody>
</table>

3.2.4 Data collection

Data was collected from hatcheries and the knowledge of MG infection and description of bio-safety procedures evaluated by questionnaire and veterinary medical certificates (See annex 1).

3.2.5 Data analysis

Data generated from the study was tabulated as Microsoft Excel sheets and uploaded to statistical package for social sciences (SPSS) version17 for windows. Cross tabulation of variables were generated. Chi square was used to detect statistically significant correlation among variables.
Chapter 4
Results

4.1 Distribution of hatchability in Gaza strip governorates

The study was conducted in ten licensed hatcheries in Gaza strip governorates. The hatchability rate during the sample collection period in August 2010 was 63% to 75% with mean value 62.2% and ± 2.78 (SE) (Figure 4.1).

Figure (4.1): Hatchability in August 2010

4.2 Samples sources

Only (15.4%) of samples were from the West Bank and (84.6%) from "Israel" derived from different parent flocks. Samples distribution according to source is illustrated in figure 4.2.

Figure (4.2): Samples distribution according to source
4.3 Eggs weight

Eggs weight ranged from 45 to 73 gm. One hundred and 4 eggs (26.7%) large, 150 (38.5%) medium and 136 (34.8%) small (Table 4.1). The egg weight categories were assigned according to table 2.4 [22].

Table (4.1): Samples distribution according to eggs weight

<table>
<thead>
<tr>
<th>Size</th>
<th>Weight</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large</td>
<td>63 – 73 gm</td>
<td>104</td>
<td>26.7</td>
</tr>
<tr>
<td>Medium</td>
<td>53 – 63 gm</td>
<td>150</td>
<td>38.5</td>
</tr>
<tr>
<td>Small</td>
<td>Less than 53 gm.</td>
<td>136</td>
<td>34.8</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>390</td>
<td>100</td>
</tr>
</tbody>
</table>

4.4 Veterinary medical certificates

4.4.1 "Israel" veterinary medical certificate

According to the "Israel" procedure, eggs were derived from parent flocks that have not been vaccinated against MG. However, the flocks had been vaccinated against other diseases such as Infectious bursal, Newcastle virus, Marek, Infectious bronchitis, Turkey rhinotracheitis, avian Influenza of hemagglutinin subtype 9 and neuraminidase subtype 2 and Inflammatory bowel diseases. In addition, the eggs have been disinfected using formalin gas for 20 minutes contact time. The eggs were tested and found negative for MG and other pathogens [Annex 3].

4.4.2 PNA veterinary medical certificate

The West Bank eggs were originated from parent flocks farms known to be free from salmonella, mycoplasma and infectious diseases and there is no information about the vaccination program from the provided certificate [Annex 4].

4.5 Data for the Prevalence of MG specific antibodies

4.5.1 Prevalence of MG specific antibodies

Out of the 390 tested eggs, 143 (36.6%) were positive, 85 (21.8%) were suspected and 162 (41.6%) were negative (Figure 4.3).
4.5.2 Prevalence of MG antibodies according to the eggs weight
According to the eggs weight, the highest positive for MG specific antibodies 71/136 (52%) was in the small samples followed by 46/150 (31%) in the medium then 26/104 (25%) in the large. There is statistically significant differences between the weight and the prevalence of MG specific antibodies (P= 0.000) (Table 4.2).

Table (4.2): Prevalence of MG specific antibodies according to eggs weight

<table>
<thead>
<tr>
<th>MG</th>
<th>Small</th>
<th>Medium</th>
<th>Large</th>
<th>Total</th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>%</td>
<td>No</td>
<td>%</td>
<td>No</td>
<td>%</td>
</tr>
<tr>
<td>Positive</td>
<td>71</td>
<td>52.2</td>
<td>46</td>
<td>31.0</td>
<td>26</td>
</tr>
<tr>
<td>Negative</td>
<td>42</td>
<td>30.8</td>
<td>58</td>
<td>38.5</td>
<td>62</td>
</tr>
<tr>
<td>Suspect</td>
<td>23</td>
<td>17.0</td>
<td>46</td>
<td>30.5</td>
<td>16</td>
</tr>
<tr>
<td>Total</td>
<td>136</td>
<td>100</td>
<td>150</td>
<td>100</td>
<td>104</td>
</tr>
</tbody>
</table>

4.5.3 Prevalence of MG antibodies according to the eggs source
Almost equal positivities for MG specific antibodies was detected in eggs imported from west bank 23/60 (38.30%) and those imported from "Israel" 120/330 (36.40%) (P. value=0.554) (Table 4.3).
Table (4.3): Prevalence of MG specific antibodies according to eggs source

<table>
<thead>
<tr>
<th>MG</th>
<th>West Bank</th>
<th>&quot;Israel&quot;</th>
<th>Total</th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>%</td>
<td>No</td>
<td>%</td>
</tr>
<tr>
<td>Positive</td>
<td>23</td>
<td>38.3</td>
<td>120</td>
<td>36.4</td>
</tr>
<tr>
<td>Negative</td>
<td>10</td>
<td>16.7</td>
<td>152</td>
<td>46.0</td>
</tr>
<tr>
<td>Suspect</td>
<td>27</td>
<td>45.0</td>
<td>58</td>
<td>17.6</td>
</tr>
<tr>
<td>Total</td>
<td>60</td>
<td>100</td>
<td>330</td>
<td>100</td>
</tr>
</tbody>
</table>

4.6 Data for the prevalence of MG-DNA

Figure 4.4 shows an example for a typical amplification plots for positive samples. In figure 4.5 there are no amplification plots for negative samples.

Figure (4.4): MG-DNA was detected in the samples
Figure (4.5): MG- DNA was not detected in the samples

4.6.1 Pooling of samples
The results did not show any difference in detection limits between the original and the pooled samples (Figures 4.6 and 4.7). Using this technique, 8 new cases of MG were detected among negative and suspected RSA test.

Figure (4.6): Results of initial experiment from pooled samples
4.6.2 Prevalence of MG-DNA

Only 24 out of the 390 samples (6.2%) were positive for MG-DNA. Internal controls were detected in all negative samples (Figure 4.8).

![Amplification Plot](image)

**Figure (4.7):** Representative results of the same non-pooled samples

![Bar Chart](image)

**Figure (4.8):** Prevalence of MG-DNA, the numbers of samples are indicated at the columns.
4.6.2.1 Prevalence of MG-DNA according to the eggs weight

The highest prevalence was 14/150 in the medium, 8/104 in the large and 2/136 in the small samples. There is statistically significant differences between the weight and the prevalence of MG-DNA (P = 0.016) (Table 4.4).

Table (4.4): Prevalence of MG-DNA according to eggs weight

<table>
<thead>
<tr>
<th></th>
<th>MG</th>
<th>Small</th>
<th></th>
<th>Medium</th>
<th></th>
<th>Large</th>
<th></th>
<th>Total</th>
<th></th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>%</td>
<td>No</td>
<td>%</td>
<td>No</td>
<td>%</td>
<td>No</td>
<td>%</td>
<td>Total</td>
<td>%</td>
</tr>
<tr>
<td>Positive</td>
<td>2</td>
<td>0.5</td>
<td>14</td>
<td>3.6</td>
<td>8</td>
<td>2.1</td>
<td>24</td>
<td>6.2</td>
<td></td>
<td>0.016</td>
</tr>
<tr>
<td>Negative</td>
<td>134</td>
<td>99.5</td>
<td>136</td>
<td>96.4</td>
<td>96</td>
<td>97.9</td>
<td>366</td>
<td>93.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>136</td>
<td>100</td>
<td>150</td>
<td>100</td>
<td>104</td>
<td>100</td>
<td>390</td>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.6.2.2 Prevalence according to the eggs source

The prevalence of MG-DNA in the samples from "Israel" was 24/390 (6.2%) and no detected samples from the West Bank (Figure 4.9).

Figure (4.9): Prevalence of MG-DNA according to eggs source, the percentage of samples are indicated at the columns.
4.7 Comparison between RSA test and real time PCR technique
Real time PCR detected 24 samples of which 16 samples were positive, 6 were negative and 2 were suspected by RSA test. The results of the two methods are summarized in table 4.5.

Table (4.5): The results by two methods

<table>
<thead>
<tr>
<th>Character</th>
<th>Positive DNA</th>
<th>Negative DNA</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive RSA</td>
<td>16</td>
<td>127</td>
<td>143</td>
</tr>
<tr>
<td>Negative RSA</td>
<td>6</td>
<td>156</td>
<td>162</td>
</tr>
<tr>
<td>Suspect RSA</td>
<td>2</td>
<td>83</td>
<td>85</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>366</td>
<td>390</td>
</tr>
</tbody>
</table>

4.8 Questionnaire
4.8.1 Knowledge of MG
Six hatchery owners claimed to have knowledge about MG. Among those, two of six stated that MG dose not affect chick health and hatchability and one of six did not think that MG demand increased medication costs. No one of them agreed that the MG has an effect on decreased eggs production, reduced feed efficiency and increased condemnations at slaughter (Table 4.6).

Table (4.6): Knowledge of MG in hatcheries (n=10)

<table>
<thead>
<tr>
<th>No</th>
<th>Questions</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Do you have information about MG?</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>The interviewed person who responded yes, do you think that?</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MG is affecting the health of chick and hatchability</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Decreased egg production</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Reduced feed efficiency</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Significant condemnations at slaughter</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Increased medication costs</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>
4.8.2 Location, design, biosafety, behavior and attitudes in hatcheries

Among the ten hatcheries, it was found that three are not separated from poultry farms and potentially contaminated areas, four were not designed according to the accepted standards of hygiene and two have no secure fence. It was found that seven have not adequate laundry, shower, and change room facilities for staff and one have no cold room for eggs. Also was found that nine have no logbooks, seven have no central points to regulate work follow and nine have no special places to eat and drink.

All hatcheries staff have not wearing a clean coveralls, laboratory coats or socks. No regular accurate sanitation programs nor special measures to assess the various processes are followed and workers did not use disposable distribution boxes. New shipments were introduced before hatching the previous ones and vehicles were not disinfected before entering the hatcheries. In addition, no hatcheries received certificates that the eggs are free of MG. They did not have knowledge about vaccines.

They did not perform routine tests for MG detection and did not send samples to a laboratory for MG examination. However all hatcheries deal with eggs which have special numbers, cleaned and disinfect the hatchery machines after each use. When there is a case of MG infection in hatcheries, all hatcheries did not get rid of eggs or of chicks. However all hatcheries owners notify the concerned authorities in a case of MG infection (Table 4.7).

Table (4.7): Location, design, biosafety, behavior and attitudes in hatcheries

<table>
<thead>
<tr>
<th>No</th>
<th>Questions</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.</td>
<td>The hatchery has a secure fence, and all entrances to the building are located inside the fenced area.</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>3.</td>
<td>The hatchery is designed according to the accepted standards of hygiene</td>
<td>6</td>
<td>4</td>
</tr>
</tbody>
</table>
Table (4.7): Location, design, biosafety, behavior and attitudes in hatcheries (Cont.,)

<table>
<thead>
<tr>
<th>No</th>
<th>Questions</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.</td>
<td>The hatchery location is separated from poultry farms and potentially contaminated areas.</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>5.</td>
<td>The hatchery has a cold room for eggs</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>6.</td>
<td>The hatchery has adequate laundry, shower, and change room facilities for staff.</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>7.</td>
<td>Are there logbooks in the hatchery to record and organizing visits between the farms?</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>8.</td>
<td>Dose the visitors wear clean coveralls (or laboratory coat), socks, and shoes provided by the hatchery?</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>9.</td>
<td>Are there central points to regulate workers follow entry and exit?</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>10.</td>
<td>Are there special places to eat and drink?</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>11.</td>
<td>The hatchery machine is cleaned and disinfected after each use?</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>12.</td>
<td>Are there accurate sanitation program implemented by specialized?</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>13.</td>
<td>Are there special measures to assess the various processes?</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>14.</td>
<td>Do use disposable distribution boxes?</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>15.</td>
<td>The hatchery adds a new shipment before hatching the previous one.</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>16.</td>
<td>All vehicles were disinfected before entering a hatchery?</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>17.</td>
<td>The hatchery receives a certificate that the eggs are free from MG?</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>18.</td>
<td>Do the hatchery deals with eggs, which have special numbers from the certified free farms?</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>19.</td>
<td>The hatchery provides vaccine against MG?</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>20.</td>
<td>Does the hatchery have routine tests for detection of MG?</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>21.</td>
<td>The hatchery sends samples to a laboratory for examination MG.</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>If case of MG infection, what do you do?</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Get rid of eggs</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Get rid of chicks.</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Both</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Notify the concerned authorities</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>
CHAPTER 5
Discussion

When one-day-old chicks are received at a farm from the hatchery, they should have a good quality; mainly, free from physical defects, actively seeking feed and water, able to respond to changes in temperature and generally exhibit normal behavior [98]. The desired levels of egg production, high internal and shell quality, optimum hatchability, and quality chicks could not be achieved when *Mycoplasma gallisepticum* (MG) infected the parent flocks. It therefore, should be routinely monitored and controlled [99].

To date, there is no data available about the prevalence of MG in Gaza strip, and the pathogen is not being tested routinely. Furthermore, fertilized eggs imported to Gaza strip from "Israel" and the West Bank have no exact data about the parents flocks. Therefore, this study focuses on determining the prevalence of MG in the ten licensed hatcheries in Gaza strip.

5.1 Hatchability

In this study, the mean values of hatchability was 62.2% ± 2.78 standard error. The relatively low percentage may be explained by that the imported eggs which were used for hatching process did not fit in the best-case specifications, in addition to the lack of proper hatchery management. In small eggs the content of solids of internal egg, eggshell quality characteristics, and inefficient yolk sac lipid mobilization and assimilation into the embryo could affect the hatchability. The total yolk lipid, cholesterol, myristate, palmitoleiate, and oleic acid percentages are significantly decreased in young hens infected with MG and linoleic acid, stearic and arachidonic acids are significantly increased [100]. In addition, the weak fertility in large eggs due to the mail aging usually decreases the hatchability. More beneficial for egg producers to use young and old birds for table egg production [98]. This result was in agreement with a study in Italy which recorded that the first egg laying presented a low hatchability (12.5%) and hatch rate remained below 50% [101].
5.2 Sample selection
Our selection of egg samples was not an option; it was necessary because of absence of the parent flocks, which produce fertilized eggs for hatching process. In Gaza strip, the selection of eggs to monitor the prevalence of MG was in part influenced by the findings that the prevalence of antibodies in eggs reflects MG infections in layers. Furthermore, there is no significant difference in level of antibodies between parent flocks serum and their eggs, and thus the eggs can be used in lieu of serum samples to screen parent flocks for the prevalence of antibodies to MG [102].

Moreover, the use of eggs in screening programs does not require syringes, blood collection tubes and needles; it avoids the expense of blood sampling and the need for trained staff. In addition, collection of eggs by farm workers also prevents potential contamination between farms or houses, which could occur during serum sampling. Sample identification can be recorded directly on the egg with a pencil and collection of eggs is not stressful for birds [103].

5.3 Prevalence of MG
Eggs of breeder flocks must be monitored for MG at regular intervals using true random sampling for early detection. The National Poultry Improvement Plan (NPIP) - approved confirmation methods of serological-based diagnoses including a polymerase chain reaction (PCR)-based procedure [99]. In this study rapid slide agglutination test (RSA) test was used to detect the prevalence of MG specific antibodies and the real time PCR technique to detect the prevalence of MG-DNA.

The results showed that the prevalence of MG specific antibodies in non-incubated imported fertilized eggs in selected hatcheries in Gaza strip was 36.6% by RSA, while the prevalence of MG-DNA was 6.2% by real time PCR and therefore, 115700 one day old chicks are expected to have the organism after the infected eggs hatch each month. It is worthy to mention that all of the tested eggs are certified for being negative for MG by the producers.
Usually MG infection of the eggs results from various reasons, such as infection of imported eggs, potentially contaminated areas in egg source, lack of accepted standards of hygiene and accurate sanitation programs and special measures to assess the various processes which could reduce the rate of infection. For comparison purpose, a number of studies from different countries, where such data were available, are listed in table 5.1.

Table (5.1): Prevalence of MG in different countries

<table>
<thead>
<tr>
<th>#</th>
<th>Place</th>
<th>Sample</th>
<th>PCR</th>
<th>RSA</th>
<th>ELISA</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Netherlands</td>
<td>Tracheal swab (hens)</td>
<td>51.6%</td>
<td>---</td>
<td>---</td>
<td>9</td>
</tr>
<tr>
<td>2.</td>
<td>France</td>
<td>Eggs</td>
<td>---</td>
<td>68.0%</td>
<td>79.0%</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>France</td>
<td>Serum (old hens)</td>
<td>---</td>
<td>93.0%</td>
<td>84.0%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>France</td>
<td>Serum (1-day old chick)</td>
<td>---</td>
<td>88.0%</td>
<td>86.0%</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Turkey</td>
<td>Tracheal swab (hens)</td>
<td>29.0%</td>
<td>---</td>
<td>---</td>
<td>12</td>
</tr>
<tr>
<td>4.</td>
<td>Italy</td>
<td>Eggs (single age hens)</td>
<td>---</td>
<td>---</td>
<td>33.3%</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>Italy</td>
<td>Eggs (multi age hens)</td>
<td>---</td>
<td>---</td>
<td>87.8%</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>USA (Florida)</td>
<td>Eggs (hens)</td>
<td>---</td>
<td>---</td>
<td>33.0%</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>USA (Florida)</td>
<td>Serum (hens)</td>
<td>---</td>
<td>---</td>
<td>31%</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Egypt</td>
<td>Serum (1-day old chick)</td>
<td>---</td>
<td>48.7%</td>
<td>60.0%</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>Egypt</td>
<td>Serum (young hens)</td>
<td>---</td>
<td>69.9%</td>
<td>58.3%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Egypt</td>
<td>Tracheal swab</td>
<td>33.3%</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>Jordan</td>
<td>Serum (hens)</td>
<td>---</td>
<td>80.4%</td>
<td>---</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>Jordan</td>
<td>Serum (hens)</td>
<td>---</td>
<td>---</td>
<td>73.5%</td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>Jordan</td>
<td>Tracheal swab (hens)</td>
<td>21.7%</td>
<td>---</td>
<td>---</td>
<td>106</td>
</tr>
<tr>
<td>9.</td>
<td>Brazil</td>
<td>Serum (hens)</td>
<td>---</td>
<td>32.8%</td>
<td>---</td>
<td>107</td>
</tr>
<tr>
<td>10.</td>
<td>Croatia</td>
<td>Serum (hens)</td>
<td>---</td>
<td>33.0%</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>Argentina</td>
<td>Serum (hens)</td>
<td>---</td>
<td>100%</td>
<td>---</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>(Victoria)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td>Argentina</td>
<td>Serum (hens)</td>
<td>---</td>
<td>8.7%</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Colon)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13.</td>
<td>Mongolia</td>
<td>Serum (hens)</td>
<td>---</td>
<td>53.0%</td>
<td>---</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>Bangladesh</td>
<td>Serum (hens)</td>
<td>---</td>
<td>58.9%</td>
<td>---</td>
<td></td>
</tr>
</tbody>
</table>
Table (5.1): Prevalence of MG in different countries (Cont.,)

<table>
<thead>
<tr>
<th>#</th>
<th>Place</th>
<th>Sample</th>
<th>PCR</th>
<th>RSA</th>
<th>ELISA</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.</td>
<td>Pakistan</td>
<td>Serum (young hens)</td>
<td>---</td>
<td>74.6%</td>
<td>---</td>
<td>110</td>
</tr>
<tr>
<td>15.</td>
<td>Pakistan</td>
<td>Serum (old hens)</td>
<td>---</td>
<td>33.1%</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>16.</td>
<td>Malaysia</td>
<td>Serum (hens)</td>
<td>---</td>
<td>26.0%</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>17.</td>
<td>Thailand</td>
<td>Serum (hens)</td>
<td>---</td>
<td>40.0%</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>18.</td>
<td>Nigeria</td>
<td>Serum (hens)</td>
<td>---</td>
<td>47.5%</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>19.</td>
<td>Venezuela</td>
<td>Serum (hens)</td>
<td>---</td>
<td>49.0%</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>20.</td>
<td>USA (Southern California)</td>
<td>Serum (hens)</td>
<td>---</td>
<td>73.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21.</td>
<td>USA (Central California)</td>
<td>Serum (hens)</td>
<td>---</td>
<td>3.0%</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>22.</td>
<td>Germany</td>
<td>Eggs (raptors)</td>
<td>0.7%</td>
<td></td>
<td>---</td>
<td>112</td>
</tr>
</tbody>
</table>

The wide variation of MG prevalence and detection rates in different studies might be due to sample size, sample type, sample time, detection techniques, rate of infection, flocks age, type of breeder hens, biosafety and biosecurity in the respective study area.

In the neighboring countries, like Egypt studies showed that MG antibody were detected in 48.7% in one-day-old chicks and 69.6% in chicken samples. This high number was attributed to cross-reactivates. In Egypt MG-DNA was relatively high (33.3%) probably due to using the tracheal swab procedure as mycoplasma infecting mucosa of respiratory system, proving an excellent method for sample collection [104].

In Jordan MG-DNA infection was (21.7%) probably because the flocks in this study were exposed to high virulent strains of MG [105]. The results were matched Saad and Dergham [113,114].

In France, MG infection in egg samples was also high (68%) probably due to selection of symptomatic cases of hens. In the other hand, the increase of MG in one-day-old chick (88%) and hens (93%) might be due to lateral
transmission of MG [9]. In USA (Central California), MG infection in egg samples was low (3%) While in southern California it was high (73%) [111].

In analogous study in Germany, the prevalence of mycoplasma infection in eggs of the bird (*Accipiter gentilis*) by using PCR technique was 0.7%. However The low MG-DNA detection in the study was explained by the poor sample quality resulting in false negative results. The infection in other tissues, particularly the genital tract was low. This low rate of transmission is still important because mycoplasma quickly spread among chicks [112].

5.3.1 Data for the prevalence of MG specific antibodies

The RSA kit was chosen based on Office International Epizootie recommendations [75]. Detection of the specific MG antibodies is not surprising particularly when the layer hens are infected with MG before or during the investigation, and infections of the oviduct with MG can result in their shedding the specific antibodies into the egg albumen [99,115,116].

5.3.1.1 Prevalence of MG antibodies according to the eggs weight

In our study there is statistically significant differences between the egg weight and the prevalence of MG specific antibodies (P= 0.000). The increase of MG specific antibodies in the small egg weight may be due to the small egg weight is a result of layer immunosuppression at time of egg production as evident from the high prevalence of MG specific antibodies in the small egg population.

Despite that, a number of large eggs were also found to be positive for specific antibodies. This may be due to extensive use of antibiotics and improvement of the layer health before disappearance of antibodies. With increasing the age, the detectable antibodies decrease, resulting from catabolism of maternal antibodies [9]. Studies showed that the presence of MG specific antibodies significantly decreases with the increase of age [109].
5.3.1.2 Prevalence according to the eggs source
In our study, the majority of egg samples were from "Israel" (84.6%) as a result of the siege imposed on Gaza strip. The presence of MG specific antibodies in the samples from the West Bank (38.3%) were correlated with those from "Israel" (36.4%), the difference was not statistically significant (P=0.554).

5.3.2 Data for the prevalence of MG-DNA
In our study, DNA extraction and purification was achieved by using optimized QIAamp MinElute DNA extraction protocol and this was very critical point to successfully obtain and ensure high quality template DNA and increase the sensitivity of detection of MG. Several investigators employed the same extraction kit and showed its high efficiency [12,112,117,118].

In addition a well optimized commercial Taqman-labeled probe (MYCO G/S real time kit) was used in this study for detection of MG-DNA. The kit has the advantage of being ready made with minimum need for pipetting, thus reducing the possibility for contamination that could emerge during the preparation of PCR reagents. Moreover, such kit was supplemented with positive control. Therefore, the commercial PCR-based test kit for the detection of MG is sensitive, specific, simple, rapid and accurate means. The use of real time PCR in this study was preferable to investigating the prevalence of MG-DNA in the eggs samples.

5.3.2.1 Prevalence of MG-DNA according to the egg weight
In this study, there is statistically significant differences between the egg weight and the prevalence of MG-DNA (P=0.016). The prevalence was 0.5%, 3.6% and 2.1% in small, medium and in large samples respectively. This distribution is different from that of MG specific antibodies in the same egg weight categories. The increase of MG specific antibodies in the small eggs with a concomitant decrease in MG organism in the same weight may be due to the effect of MG specific antibodies obliteration of MG organism.
The MG maternal antibodies prevent or significantly reduce embryo mortality caused by MG and reduce replication of MG and the primary means of antigen-specific protection [23,119].

5.3.2.2 Prevalence according to the eggs source
The prevalence of MG-DNA in the samples from "Israel" (6.2%) is a direct result of the vertical transmission of MG organism. None detected samples from the West Bank and "Israel" may have resulted from that the eggs were derived from MG free parent flocks or the eggs were exposed to disinfectants such as formalin gas for 20 minutes (claimed by the producers). The use of medication, the influence of summer season weather, the presence of MG maternal antibodies, the absence of multiplication in egg and not enough MG cells have affected the result. This claim is supported by others [2,12,106,108].

When comparing the results of RSA and DNA detection, 14, 2 and 6 samples (positive, suspect and negative respectively) were found positive among RSA samples. Real time PCR eliminates the disadvantages of serological false positive and false-negative reactions. The positive MG specific antibodies should be considered presumptive for the presence of MG pathogen and eggs testing positive by this test should be considered as potential carriers of MG and should not be used in relocation efforts [77]. The real time PCR technique indicates the presence of the organism and can consequently be interpreted as the detection of infected eggs [12]. Both methods, therefore are effective in detecting the presence of MG, with varying degrees of success and discrepancy.

5.4 Questionnaire
In Gaza strip hatcheries, the knowledge about MG pathogen was good. However, the lack of scientific workshops, visits, absence of technical experience, education, inspector visits and control programs and poor performance of the relevant authorities worse the situation.
In addition, the disturbance in location, design, behavior and attitudes in hatcheries reduced the use of control measures. The infection of MG in the present study, indicates that the biosecurity procedures might be poor and the vertical transmission of MG diseases can be transmitted to hatcheries through the border checkpoints. The special measures to assess the various processes according to the accepted standards of the management and control, helps elimination the disease agents [108].

The successful of MG control begins with the using of MG-free breeding flock, and strict biosecurity is invariably required to avoid the infection of MG [120]. United States department of agriculture veterinary services recorded the low prevalence of MG in some countries is probably due to the intense control, which has been conducted for several years [121]. In Gaza strip hatcheries when there is a cases of MG infection, all hatcheries do not get rid of eggs and chicks. This is probably due to the absence of compensation from veterinary services to comfort the hatchery owner.
Chapter 6
Conclusions and Recommendations

6.1. Conclusions
The poultry industry in Gaza strip is facing the infection of non-incubated imported fertilized eggs in hatcheries with *Mycoplasma gallisepticum* (MG). The disease causes decreased hatchability in 10 hatcheries included in the study. Findings from this study may be summarized as follows:

1. The prevalence of MG specific antibodies in non-incubated imported fertilized eggs in selected hatcheries in Gaza strip was 36.6% by RSA while the prevalence of MG-DNA was 6.2% by real time PCR.
2. Real time PCR technique had detected 2 and 6 of suspected and negative MG specific antibodies respectively.
3. Our results showed that there is significant correlation between the eggs weight and the prevalence of MG specific antibodies (P= 0.000) and between the eggs weight and the prevalence of MG-DNA (P= 0.016). There is no significant correlation between the sources and the prevalence of MG (P= 0.554). The prevalence of MG specific antibodies was highest in the small samples, while the prevalence of MG-DNA was the lowest in the same samples.
4. Reliance on visual observation and certification assessment in border checkpoints is important but not enough for prevention of MG transmission.
5. The eggs sources which are used in Gaza strip hatcheries are not from local layer flocks and the detection of MG infection is not possible from the layers, thus the direct egg examination is the method of choice.
6. The current quality measures and procedures for control of MG infections in local hatcheries are limited or absent.
7. Applying pooling strategy for the prevalence of microorganism such as MG is effective and lowers the expenses associated with real time PCR.
8. Real time PCR technique is the method of choice for screening the presence of MG pathogen while RSA test may be used to assess the infection in mothers layers.

6.2. Recommendations

6.2.1. Recommendations from the study

1. Changing the poultry handling strategy in Gaza strip by breeding parent flocks to produce fertilized eggs.
2. Raising awareness of workers, farmers and drivers on the importance of special measures to assess the various processes according to the accepted standards of the management and control, resistance of drugs, effect of vaccination and dealing with disease.
3. We strongly recommend government veterinary services not to neglect MG in the imported eggs and private veterinarians to determine the etiology of chronic respiratory disease before prescribing antibiotics and preventing antibiotics used for human therapy to be used for poultry medication.
4. We recommend the Palestinian official veterinary services certify that the chicks, which are produced in the hatcheries, are free of MG.
5. We recommend real time PCR technique to be used along with MG RSA test.
6. The losses caused by MG were not estimated in this study. Further research is necessary to assess the economic impact of MG on the poultry production.

6.2.2. Recommendations from the literature

1. Breeders should first medicate then use vaccine in breeders which will improve the quality of progeny and the hatching of eggs.
2. Large extent quality management for each step of egg importation and egg management practices should be performed to increase profitability.
3. For possible corrective measures for the ongoing infection with MG in Gaza strip hatcheries see annex 5.
References


[75] Avian mycoplasmosis (Mycoplasma gallisepticum and Mycoplasma synoviae): http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.03.05_%20AVIAN_MYCO.pdf, (Last access on 22/1/2011).


[96] **Survey random sample calculator:**


[107] Talha A., 2003- Investigation on the prevalence and significance of Mycoplasma gallisepticum in layer chickens in Bangladesh. The royal vet. and agricult. Univ. (Un published data)


Annexes
Annex 1

العنوان

اسم القائمة

المساحة الكلية

السعة الكلية

لا نعم □ □

1. هل لديك معلومات عن الميكوبلازما غالابسينكم؟
   □ 1. تثير ON 창구 الصويس ونسبة القفس
   □ 2. تقييم تكافح بالعلاف
   □ 3. تقييم كفاءة الأدوات

التوزيع الدموغرافي والسلامة البيولوجية

2. القائمة لها سياج أمن، وجميع المداخل المؤدية إلى المبنى موجودا داخل المنطقة المحاطة بالسياج؟
   □ نعم □ لا

3. تصميم القائمة ضمن المعايير الصحية المسموح بها؟
   □ نعم □ لا

4. موقع القائمة بعيدا عن مزارع الدواجن والمناطق المحتمل أن تكون ملوثة؟
   □ نعم □ لا

5. يوجد غزفة مبردة للخزين؛
   □ نعم □ لا

6. يوجد مراقب لللاستفاح وغسل الملابس وتقديم الملابس متاحة للعاملين؟
   □ نعم □ لا

7. يوجد سجلات في القائمة لتسجيل وتنظيم الزيارات بين المزارع؟
   □ نعم □ لا

8. يرتمي الزوار ملابس واقية واحدة يتم توفيرها من القائمة؟
   □ نعم □ لا

9. يوجد نقطة رئيسية لتنظيم أشياء العمل في الدخول والخروج؟
   □ نعم □ لا

10. يوجد مراقب خاصة للتغذية والشراب؟
    □ نعم □ لا

11. يتم تنظيف ماكينات القائمة بعد كل استخدام؟
    □ نعم □ لا

12. يوجد برنامج صحي دقيق ينفذ من خلال مختصين؟
    □ نعم □ لا

13. يوجد معايير خاصة لتقسيم العمال المختلفة في القائمة؟
    □ نعم □ لا

14. هل تستخدم القائمة صناديق التوزيع أحادية الاستعمال؟
    □ نعم □ لا
- 15. هل تقدم الغطاسة شحنات جديدة قبل نفس الشحنة السابقة؟
  - نعم □ لا □

- 16. جميع المنتجات يتم تقديمها قبل دخولها الغطاسة؟
  - نعم □ لا □

- 17. تستلم الغطاسة شهادة تفيد أن البيض من الميكوبلازما غالاسيتكم؟
  - نعم □ لا □

- 18. هل الغطاسة تتعامل مع بيض يحمل أرقام خاصة من مزارع خالية من المرض؟
  - نعم □ لا □

- 19. الغطاسة توفر تحصين ضد مرض الميكوبلازما غالاسيتكم؟
  - نعم □ لا □

- 20. هل يوجد فحوصات روتينية للكشف عن الميكوبلازما غالاسيتكم؟
  - نعم □ لا □

- 21. هل يتم إرسال عينات للمختبر البيطرى لإجراء فحوصات الميكوبلازما غالاسيتكم؟
  - نعم □ لا □

- 22. في حال وجود عدوى في الفحص ماذا تفعل؟
  - التخلص من البيض □
  - التخلص من الصوص □
  - كلاهما □
  - إعلام الجهات المختصة □
Annex 2

An English version questionnaire
This questionnaire about the occurrence of *Mycoplasma gallisepticum* (MG) in hatcheries in Gaza strip submitted as part of the requirements for the degree of Master of biological sciences Islamic university – Gaza.

Hatchery name .......................... Address ..........................
Total capacity .......................... Total area ..........................

1- Do you have any information about MG? □ No □ Yes

If yes, what do you know?
1- MG is affecting the health of chick and hatchability { } { }
2- decreased egg production { } { }
3- reduced feed efficiency { } { }
4- significant condemnations at slaughter { } { }
5- Increased medication costs { } { }

Demographic characters
2- The hatchery has a secure fence, and all entrances to the building are located inside the fenced area □ No □ Yes

3- The hatchery is designed according to the accepted standards of hygiene. □ No □ Yes

4- The hatchery location is separated from poultry farms and potentially contaminated areas. □ No □ Yes

5- The hatchery has a cold room for eggs. □ No □ Yes

6- The hatchery has adequate laundry, shower, and change room facilities for staff. □ No □ Yes

7- Are there logbooks in the hatchery to record and organizing visits between the farms? □ No □ Yes

8- Does the visitors wear clean coveralls (or laboratory coat), socks, and shoes provided by the hatchery? □ No □ Yes

9- Are there central points to regulate workers follow entry and exit? □ No □ Yes

10- Are there special places to eat and drink? □ No □ Yes

11- The hatchery machine is cleaned and disinfected after each use □ No □ Yes
12- Are there accurate sanitation program implemented by specialized?

☐ No ☐ Yes

13- Are there special measures to assess the various processes?

☐ No ☐ Yes

14- Do use disposable distribution boxes?

☐ No ☐ Yes

15- The hatchery adds a new shipment before hatching the previous one.

☐ No ☐ Yes

16- All vehicles are disinfected before entering a hatchery.

☐ No ☐ Yes

17- The hatchery receives a certificate that the eggs are free from MG?

☐ No ☐ Yes

18- Does the hatchery deals with eggs, which have special numbers from the certified free farms?

☐ No ☐ Yes

19- The hatchery provides vaccine against MG?

☐ No ☐ Yes

20- Does the hatchery has routine tests for detection of MG?

☐ No ☐ Yes

21- The hatchery sends samples to a laboratory for examination MG

If yes, which parts are tested?

1-Incoming eggs { } 6-Setters { }
2-The egg room { } 7-Hatchers after disinfection { }
3-Hatcher rooms { } 8-Setters rooms { }
4-Chick-processing rooms { } 9-The water supply { }
5-Vehicles { } 10-Exhaust ducts { }

22- If case of MG infection, what do you do?

1- Get rid of eggs. { }
2- Get rid of chicks. { }
3- Both. { }
4- Notify the concerned authorities { }
Annex 3

"Israel" veterinary medical certificate

STATE OF ISRAEL
MINISTRY OF AGRICULTURE AND RURAL DEVELOPMENT
VETERINARY SERVICES & ANIMAL HEALTH
VETERINARY CERTIFICATE
for hatching eggs of poultry other than ratites (HEP)

<table>
<thead>
<tr>
<th>DUNTRY</th>
<th>ISRAEL</th>
<th>Veterinary certificate to Palestinian Authority</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1. Consignor:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Name:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Address:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tel. No:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.2. Certificate reference number:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.3. Central Competent Authority: Israel Veterinary Services Ministry of agriculture and Rural Development</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.4. Local Competent Authority: Israel Veterinary services:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5. Consignment Name: HACHERY</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Address:</td>
<td></td>
<td></td>
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<tr>
<td>Postal code</td>
<td></td>
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<tr>
<td>Tel. No:</td>
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<td>1.6.</td>
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<tr>
<td>ISRAEL IL</td>
<td></td>
<td></td>
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<tr>
<td>Palestinian Authority PS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.11. Place of origin Name: Approval number: Address:</td>
<td></td>
<td></td>
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<tr>
<td>1.12.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.13. Place of loading: Approval number: Address:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.14. Date of departure: time of departure:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.15. Means of transport Airplane: Ship: Railway wagon: Road vehicle: Other:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Identification:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Documentary references:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.16.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.17. Number of packages:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.18. Description of commodity Hatching eggs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.19. Commodity code (HS code):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.20. Quantity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.21.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.22. Number of packages</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.23. Identification of container/totol number</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.24. Commodity certified for: Breeding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.26.</td>
<td></td>
<td></td>
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<tr>
<td>1.27. For import or admision:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.28. Identification of the commodities</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species (Scientific name): Gallus gallus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breed/Category: Rose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Identification system: Stamp on eggs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Identification number</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quantity</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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II. Health information

II.1. Animal health attestation

I, the undersigned official veterinarian, hereby certify that the hatching eggs (?) described in this certificate:

II.1.1. meet the provisions of Directive 90/659/EEC;

II.1.2. come from flocks which have remained on:

(?) either [the territory of code IL.0]

(?) or [department(s)]

for at least three months. Where the flocks from which the hatching eggs come were imported into the country, territory, zone or compartment of origin, this took place in accordance with veterinary conditions at least as strict as the relevant requirements of Directive 90/659/EEC and any subsidiary Decisions;

II.1.3. come from:

(?) either [the territory of code IL.0]

(a) which, at the date of issue of this certificate, was (were) free from Newcastle disease as defined in Regulation (EC) No...

(b) where a surveillance programme for avian influenza according to Regulation (EC) No... is carried out;

II.1.4. come from:

(?) either [the territory of code IL.0]

(?) or [department(s)]

(?) either [II.1.4.1 which, at the date of issue of this certificate was (were) free from highly pathogenic and low pathogenic avian influenza as defined in Regulation (EC) No...]

(?) or [II.1.4.1 which, at the date of issue of this certificate was (were) free from highly pathogenic avian influenza as defined in Regulation (EC) No...]

(?) either (a) were derived from parent flocks which have been kept in an establishment in which avian influenza surveillance has been carried out with negative results within 21 days prior to the time of collection of eggs;

(b) the hatching eggs come from an establishment:

— around which within a 1-km radius low pathogenic avian influenza has not been present within the last 30 days on any establishment;

— where there has been no epidemiological link to an establishment where avian influenza has been detected within the last 30 days;

II.2.15. were derived from parent flocks which:

(?) either [have not been vaccinated against avian influenza]

(?) or [have been vaccinated against avian influenza in accordance with a vaccination plan under Regulation (EC) No... using...]

(name and type of used vaccine(s))

(at the age of... weeks)
come from flocks which:

(a) have been examined at the date of issue of this certificate and showed no clinical signs of or grounds for suspecting any disease;

(b) have been kept for at least six weeks immediately prior to import to the Community in the establishment(s) defined in Box I.II of Part I, officially approved in accordance with requirements that are at least equivalent to those laid down in Annex II to Directive 90/539/EEC:
   — the approval of which has not been suspended or withdrawn;
   — which is (are) not subject to any animal health restriction;
   — within a 10 km radius of which, including, where appropriate, the territory of a neighbouring country, there has been no outbreak of highly pathogenic avian influenza or Newcastle disease for at least the previous 30 days;

(c) during the period mentioned in (b), have had no contact with poultry not meeting the requirements laid down in this certificate or with wild birds;

(d) have undergone a disease surveillance programme for:
   — *Salmonella* pullorum, *S. gallinarum* and *Mycoplasma gallisepticum* (fowls);
   — *Salmonella* arizonae, *S. pullorum* and *S. gallinarum*, *Mycoplasma meleagridis* and *M. gallisepticum* (turkeys);
   — *Salmonella pullorum* and *S. gallinarum* (guinea fowls, quails, pheasants, partridges and ducks)

in accordance with Chapter III of Annex II to Directive 90/539/EEC and were not found to be infected, or showed any grounds for suspecting infection, by these agents;

(1) either

(2) or

(3) or

(4) have not been vaccinated against Newcastle disease(s);

(5) have been vaccinated against Newcastle disease using:

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Live at the age of</th>
<th>Inactivated at the age of</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 weeks</td>
<td></td>
<td></td>
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<tr>
<td>2 weeks</td>
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<td></td>
</tr>
<tr>
<td>4 weeks</td>
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<td></td>
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<tr>
<td>9 weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19 weeks</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(6) have been vaccinated using officially approved vaccines

- on 1 weeks against Marek disease (repeat as necessary)
- on 1, 2, 3, 15, 16 weeks against IB.
- on 1, 7, 12, 18 weeks against *Salmonella*
- on 4, 12, 19 weeks against TRT
- on 25, 12, 18 weeks against H9N2
- on 6 weeks against IB.D
- on 2, 4, 12, 18 weeks against REO
- on 4, 18 weeks against FOWL Pox
- on 14 weeks against L.T.
- on 19 weeks against A.E.

(7) have been marked as indicated in point 1.26 of the certificate using black (colour ink)

(8) have been disinfected in accordance with my instructions, using FORMALIN GAS (name of the product and active substance) for 20 minutes (time in minutes)
were collected from _______ to _______. (dd/mm/yyyy);

have been examined at the date of issue of this certificate and showed no clinical signs of or grounds for suspecting any disease.

II.2. Public health additional guarantee

The Salmonella control programme referred to in Article 10 of Regulation (EC) No 2100/2003 and the specific requirements for the use of antimicrobials and vaccines in Regulation (EC) No 1772/2003, have been applied to the parent flock of origin and this parent flock has been tested for Salmonella serotypes of public health significance.

Date of last sampling of the parent stock from which the testing result is know

Result of all testing in the parent flock:

(+) either (positive)

(-) or (negative)

Neither Salmonella Enteritidis nor Salmonella Typhimurium were detected within the control programme referred to in point II.2.1.

II.3. Animal health additional guarantee

I, the undersigned official veterinarian, further certify that:

(+) II.3.1 where the consignment is intended for a Member State the status of which has been established in accordance with Article 13(3) of Directive 90/434/EEC, the hatching eggs described in this certificate are derived from poultry which:

(+) either (have not been vaccinated against Newcastle disease)

(+) or (have been vaccinated against Newcastle disease using non-inactivated vaccines)

(+) or (have vaccinated against Newcastle disease using a live vaccine at the latest 60 days before the date the egg were collected)

(+) II.3.2 the following additional guarantees, laid down by the Member State of destination in accordance with Articles 13 and/or 14 of Directive 92/65/EEC, are provided:

(+) if the Member State of destination is Finland or Sweden, the hatching eggs come from flocks which have tested negative in accordance with the rate laid down in Commission Decision 2003/544/EC

II.4. Additional health requirements

I, the undersigned official veterinarian, further certify that:

(+) II.4.1 although the use of vaccines against Newcastle disease which do not fulfill the specific requirements of Annex VI (II) to Regulation (EC) No 769/2008 is not prohibited in

(+) either the territory of code

(+) or compartment(s)

the poultry from which the hatching eggs are derived:

(a) has not been vaccinated for at least the previous 13 months with such vaccines;

(b) comes from a flock or flocks that underwent a virus isolation test for Newcastle disease, carried out in an official laboratory not earlier than 14 days preceding consignment on a random sample of cloacal swabs from at least 60 birds in each flock concerned and in which no avian paramyxoviruses with an Influenza Pathogenicity Index (IPI) of more than 0,4 have been found;

(c) has been kept in isolation during the last 60 days before consignment with poultry that does not fulfill the conditions in (a) and (b);

(d) has been kept in isolation under official surveillance on the establishment of origin in the 14-day period mentioned in (b);

II.5. Animal transport attestation

I, the undersigned official veterinarian, further certify that:

II.5.1 the hatching eggs are transported in perfectly clean disposable boxes used for the first time and which:

(a) contain only hatching eggs of the same species, category and type coming from the same establishment;

(b) bear the following indications:

— the word 'hatching';

— the name of the country, territory, zone or compartment of consignment,

— the species of poultry concerned.
Certificate reference number:

---
- the number of eggs,
- the category and type of production for which they are intended,
- the name, address and approval number of the production establishment,
- the approval number of the establishment of origin,
- the Member State of destination;

(c) are closed in accordance with the instructions of the competent authority to avoid any possibility of substitution of the contents;

II.5.2

the containers and vehicles in which the boxes mentioned above have been transported have been cleaned and disinfected before loading in accordance with the instructions of the competent authority.

Notes

Part I:
- Box 1.18 provide the code for the zone or the compartment of origin, if necessary, as defined under code in columns 2 of Part 1 of Annex I to Regulation (EC) No 799/2008
- Box 1.11: Name, address and approval number of the breeding establishment.
- Box 1.15: Indicate the registration number(s) of railway wagon(s) and boxes, the names of ships and, if necessary, the flight numbers of aircraft. In the case of transport in containers or boxes, the total number of boxes and their registration and where there is a serial number of the one that has to be indicated in box 1.3.
- Box 1.28 (Category): select one of the following: Pure line/parent stock/petreating pullets/eggs of turkeys for consumption/other; (identification system & identification number) introduce the egg mark.

Part II:

(1) For hatching eggs of poultry as defined in Regulation (EC) No 799/2008 with the exception of turkeys.
(3) Keep as appropriate.
(4) Insert the name of compartment(s).
(5) Apply to the poultry which belongs to the species Gallus gallus.
(6) If any of the results were positive for the following encephalomyelitis during the life of the parent flock, indicate as positive: Salmonella infantis, Salmonella Enteritidis and Salmonella Hadar.
(7) To delete if consignment is not intended for Finland and Sweden.
(8) Keep if appropriate.
(9) At the time of consignment the eggs must be individually marked in accordance with Commission Regulation (EEC) No 1598/77, including the approval number of the breeding establishment, in indelible black ink; such markings must be in legible writing and in at least one Community language.
(10) For countries or territories with the entry N° in column 6 of Part 1 of Annex I to Regulation (EC) No 799/2008, for hatching eggs of poultry other than turkeys (HEP) only, this means that in the case of an outbreak of Newcastle disease as defined in Regulation (EC) No 799/2008 then the country code or territory code shall continue to be used but this will exclude any area under official restrictions, by the third country concerned in relation to Newcastle disease, at the date of issue of this certificate.
(11) For countries or territories with the entry L° in column 6 of Part 1 of Annex I to Regulation (EC) No 799/2008, for hatching eggs of poultry other than turkeys (HEP) only, this means that in the case of an outbreak of a notifiable disease as defined in Regulation (EC) No 799/2008 then the country code or territory code shall continue to be used but this will exclude any area under official restrictions, by the third country concerned in relation to Newcastle disease, at the date of issue of this certificate.

This certificate is valid for 10 days.

Done at ......................................................... on .........................................................

(Place) (Date)

..........................................................

(Signature of poultry disease officer)

..........................................................

(Handwritten text)
Annex 4

PNA veterinary medical certificate

Palestinian National Authority
MINISTRY OF AGRICULTURE
Directorate of Veterinary Services & Animal Health

Date:
No.: 

Veterinary Medical Certificate

To:

his is to certify that the (eggs)

Accompanying this certificate are originated from parent stock farm
Known to be free from salmonella mycoplasma & infectious diseases.

Upon the company's request they were given this certificate.

Dr.
Manager of veterinary Dept.
Annex 5

Possible corrective measures for the ongoing infection with MG

The hatchery must receive certificate from official veterinary services and animal health from export country certifies that the origin of eggs shipment were come from flocks were found free from evidence of chronic respiratory disease [2]. Reusable plastic egg trays should be cleaned and disinfected after each use. Since fiber trays and cardboard boxes cannot be cleaned and disinfected, these should not be reused. A dedicated egg-collection vehicle should be graded and decontaminated before entering a hatchery. Washing and disinfection of vehicles should be possible at the hatchery too [3]. It is not allowed to use the same driver and truck for the transfer both chicks and eggs, or vice versa [1].

The hatchery should have a quality control program to monitor incoming eggs visually and microbiologically [3]. Single-stage setters should be cleaned and disinfected after each transfer. Broken eggs should be removed from setters daily. Setter rooms should be disinfected daily. Setter racks should be cleaned and inspected before return to the breeding farm [1].

Certain types of disinfectants/ sanitizers are better suited for hatcheries, others for housing and others for vehicles. The disinfectants typically found in hatchery are halogens, chlorine, iodine, quaternary ammonium, phenols, alkylating agents, formaldehyde, gluteraldehyde, oxidizing agents and ozone, hydrogen peroxide. The disinfectants normally not used are creosols, dyes, gentian violet - blocks cell wall synthesis, Heavy metals, silver - silver nitrate, copper - copper sulfate, mercury – merthiolate, Alcohol, Radiation [4].

Quality control procedures include examination of hatchery fluff, agar gel impression disks, exposure of media plates to air, centrifugal air sampling, and surface swabs. Routine tests should include incoming eggs, the egg room, setters and hatchers after disinfection, hatcher rooms, setter's rooms, chick-processing rooms, vehicles, exhaust ducts, and the water supply. [1]. To reduces egg transmission of MG a greatly method when eggs warmed to 37.8 C and immersed in cold (1.7 to 4.4 °C) antibiotic solution (tylosin, erythromycin or gentamycin) for 15 – 20 mins. [5].

Eggs must be treated by a spraying or fumigation using formaldehyde and carbon dioxide permanganate according to international standards. About 53 cm³ were mixed from formalin and 35 grams permanganate for each square meter for 20 minutes at room temperature in the presence of a fan. Chick boxes should be cleaned on return to the hatchery. At the end of each hatching day, the chick-processing room should be cleaned and disinfected,
including all work surfaces. One central point of entry adjacent to the change room should be designated. An appropriate cleaning and disinfection program should be followed, in accordance with the recommendations of suppliers of chemicals and equipment [1].

**All hatchery personnel** should follow strict hygiene guidelines: All employees must park in a designated area. They should not be visiting other poultry facilities. Under the rare occasion when this might occur, they would have to take a shower and use clean clothes before re-entering the hatchery. They should use protective clothing, including boots, only used at the hatchery. If other company personnel need access to the hatchery, they should not have been in contact with poultry at least 24 hours prior to the visit [3].

The hatchery sanitation program should be incorporated into a hatchery manual. Procedures should specify disinfectants, concentration, and the method and frequency of application. Procedures should be reviewed and updated as necessary. Only properly trained personnel should clean and apply disinfectants. Routine monitoring of cleaning and disinfection should be carried out and appropriate remedial action should be taken [1].

**Visitors** should wear clean coveralls (or laboratory coat), socks, and shoes provided by the hatchery. If the hatchery cannot provide shoes or boots, it should have disposable plastic boots available. Once in the hatchery, visitors must follow the same rules as the employees [3]. A logbook should be kept for entry of visitors or deliveries to the hatchery, recording date and time and the previous farm or site visited. Visiting the hatcheries by the staffs should be limited to one hatchery per day [3]. To minimize contamination from one room to the next, positive pressure rooms are important in critical areas so that contamination will not be drawn in through an open door. Doors help stop cross contamination between rooms. One-way doors can be installed to increase compliance by workers. Workflow must be monitored and controlled by Management [3].