# 3. MATERIAL AND METHODS

## 3.1. MATERIAL

### 3.1.1. Samples (chickens):

Samples were collected from 55 chicken flocks which represented 39 layers and 16 breeders belonging to El-Sharqia (22), El-Gharbia (14), El-Qalubia (11) and El-Minofia (8) governorates, Egypt during the period from September 2016 till August 2017, and investigated for presence of *P. multocida* infection **(Table 1)**.

### Collection of Samples:

Six chickens were taken from each flock suspected to be infected with FC. The total number of birds was 330 that represented 234 and 96 freshly dead layers and breeders chickens, respectively. Samples were taken from birds showing signs of septicaemia (congestion and cyanosis of comb and wattles), nasal and ocular discharge, conjunctivitis, greenish diarrhea, and increased mortality (5-10%), and also samples were taken from freshly dead birds with severe septicaemia, spleen congestion, liver necrosis, fibrinous perihepatitis and fibrinous pericarditis.

Specimens including liver, heart and spleen that were taken from freshly dead birds were uniquely identified and rapidly transported in sterile plastic bags to the laboratory and kept in refrigerator at 2-5$^{o}$C till examination.

Blood films were taken from living chickens with septicaemia. Moreover, liver impression smears from dead chicken were collected for staining.

**Table (1): Total number of examined flocks distributed in different Egyptian governorates**

| Governorate | Number of farms | Type of production | |
|---|---|---|---|
| | | Layers | Breeders |
| **El-Sharqia** | 22 | 15 | 7 |
| **El-Gharbia** | 14 | 10 | 4 |
| **El-Qalubia** | 11 | 8 | 3 |
| **El-Minofia** | 8 | 6 | 2 |
| **Total** | **55** | **39** | **16** |

The history of FC suspected flocks in each governorate (type of production, age/week, number of chickens in each flock and vaccination) is presented in **tables 2, 3, 4 and 5**.

**Table (2): The history of FC suspected flocks in El-Sharqia governorate**

| Farm number | Type of production | Age /week | Number of chickens/flock | *Vaccination |
|---|---|---|---|---|
| 1 | Layers | 25 | 7000 | - |
| 2 | Layers | 28 | 15500 | - |
| 3 | Layers | 38 | 10300 | - |
| 4 | Layers | 39 | 9000 | + |
| 5 | Layers | 40 | 13245 | - |
| 6 | Layers | 31 | 5320 | - |
| 7 | Layers | 26 | 4000 | + |
| 8 | Layers | 32 | 15000 | + |
| 9 | Layers | 90 | 19000 | + |
| 10 | Layers | 43 | 19000 | - |
| 11 | Layers | 40 | 15000 | + |
| 12 | Layers | 46 | 17200 | - |
| 13 | Layers | 25 | 14400 | - |
| 14 | Layers | 26 | 14000 | - |
| 15 | Layers | 43 | 17000 | + |
| 16 | Breeders | 28 | 14000 | - |
| 17 | Breeders | 48 | 11000 | + |
| 18 | Breeders | 32 | 50001 | + |
| 19 | Breeders | 64 | 13600 | + |
| 20 | Breeders | 29 | 19000 | - |
| 21 | Breeders | 21 | 10000 | + |
| 22 | Breeders | 33 | 17500 | - |

**Table (3): The history of FC suspected flocks in El-Gharbia governorate**

| Farm number | Type of production | Age /week | Number of chickens/flock | *Vaccination |
|---|---|---|---|---|
| 23 | Layers | 20 | 18000 | + |
| 24 | Layers | 21 | 17500 | - |
| 25 | Layers | 40 | 17500 | - |
| 26 | Layers | 16 | 16000 | + |
| 27 | Layers | 29 | 16000 | + |
| 28 | Layers | 62 | 16500 | - |
| 29 | Layers | 18 | 12000 | + |
| 30 | Layers | 18 | 20000 | - |
| 31 | Layers | 24 | 15000 | + |
| 32 | Layers | 25 | 16000 | - |
| 33 | Breeders | 38 | 17000 | + |
| 34 | Breeders | 35 | 15600 | + |
| 35 | Breeders | 47 | 16500 | + |
| 36 | Breeders | 50 | 13000 | + |

**Table (4): The history of FC suspected flocks in El-Qalubia governorate**

| Farm number | Type of production | Age /week | Number of chickens/flock | *Vaccination |
|---|---|---|---|---|
| 37 | Layers | 79 | 15000 | + |
| 38 | Layers | 10 | 10000 | - |
| 39 | Layers | 53 | 15000 | + |
| 40 | Layers | 21 | 12000 | + |
| 41 | Layers | 117 | 17000 | + |
| 42 | Layers | 28 | 14000 | - |
| 43 | Layers | 9 | 16000 | - |
| 44 | Layers | 24 | 19000 | - |
| 45 | Breeders | 24 | 14000 | + |
| 46 | Breeders | 43 | 23000 | + |
| 47 | Breeders | 26 | 18500 | + |

**Table (5): The history of FC suspected flocks in El-Minofia governorate**

| Farm number | Type of production | Age /week | Number of chickens/flock | *Vaccination |
|---|---|---|---|---|
| 48 | Layers | 27 | 16000 | + |
| 49 | Layers | 10 | 18500 | - |
| 50 | Layers | 31 | 18000 | + |
| 51 | Layers | 28 | 19000 | + |
| 52 | Layers | 22 | 16000 | - |
| 53 | Layers | 28 | 14400 | + |
| 54 | Breeders | 98 | 19000 | + |
| 55 | Breeders | 39 | 16500 | + |

**(+)** = vaccinated **(-)** = not vaccinated

*Vaccination against FC was done using inactivated oil adjuvant bacterin prepared from local *P. multocida* serotypes A: 1, 3 and 4 strains. Chickens were vaccinated I/M with 0.5 ml at 8-10 weeks old and boostered at 16-17 weeks old.

### 3.1.2. Bacteriological media:

#### 3.1.2.1. Media used for cultivation and isolation of *Pasteurellaspp*:

##### 3.1.2.1.1. Liquid media:

**A. Trypticase soy broth (Oxoid)**

**B. Brain heart infusion broth (Oxoid)**

They were used for the cultivation of *Pasteurella*spp. isolates.

##### 3.1.2.1.2. Solid media:

**A. Blood agar medium (Mackie and MacCartney, 1996)**

Blood agar base **(Oxoid)** plus 5-7% sheep blood was used as enriched medium for isolation of *Pasteurella*spp.

**B. Modified Das medium (Das, 1958):**

It was used as a selective and differential medium for the isolation of *P. multocida* due to their ability to grow in the presence of 0.1% crystal violet.

-It is composed of:

| | |
|---|---|
| Blood agar base | 40gm / liter |
| 0.1% crystal violet solution | 1 ml |
| 1.0% cobalt chloride solution | 3 ml |
| Esculine | 1 gm |
| pH | 7.4 |

The blood agar was prepared, autoclaved at 121ºC for 15 min. and adjusted pH to 7.4. Sterile solution of crystal violet, cobalt chloride and Esculine were added. The mixture was sterilized by steaming at 100ºC for one hr. After cooling at 45ºC, 5% sheep blood agar was added.

**C. Brain heart infusion agar (Oxoid):**

It was used for isolation of *P. multocida*.

**D. MacConkey's agar medium (Oxoid):**

It was used as an indicator and differential medium for detection of enteric contaminants and differentiation between *P. multocida* and organisms of family *Enterobacteriaceae*.

**E. 0.4 soft agar (Mackie and MacCartney, 1996)**

It is consisted of 0.4% agar powder dissolved in nutrient broth for preservation of *Pasteurella* spp.

**3.1.2.2. Media used for biochemical identification of *P. multocida*:**

**1. Buffer peptone water 2% (Oxoid):**

It was used as a base of indole production test and sugar fermentation test.

**2. Christensen's urea agar base (Himedia):**

It was used as a base in urea hydrolysis test.

**3. Sugar media:**

1% peptone water **(Oxoid)** containing Andrade's indicator was added to 1% of solution of the following sugars: fructose, glucose, sucrose, lactose maltose, mannitol, dulcitol, xylose and arabinose. One Durham's tube was introduced in an inverted position in each sugar medium tube.

**4. Triple sugar iron agar (Oxoid):**

It was used for detection of hydrogen sulfide production and fermentation of glucose, lactose and sucrose.

**5. Nitrate broth medium (Merck):**

It was used for nitrate reduction test.

**3.1.2.3. Media used for testing the sterility of prepared vaccine (OIE, 2013)**

1. Fluid thioglycollate media (FTM) **(Oxoid)** for detection of aerobic bacterial contamination.
2. Fluid thioglycollate media with 0.5% beef extract (FTMB) **(Oxoid)**, for detection of anaerobic bacterial contamination.
3. Soybean casein digest medium (SCDM) **(Difco)** for detection of fungal contamination.

**3.1.3. Reagents and solutions used for biochemical identification:**

**1. Oxidase reagent** (**Himedia):**

Standard oxidase discs impregnated with tetramethyl-P-phenylene-diamine dihydrochloride were used to determine the presence of oxidase enzyme.

**2. Hydrogen peroxide solution 3%:**

It was used for detection the presence of catalase enzyme.

**3. Kovac's reagent (Mackie and MacCartney, 1996):**

It was used for indole test.

**4. Urea solution 40% (Sigma)**.

It was used for urease test.

**5. Reagents for nitrate reduction test (Sigma):**

- Sulphalinic acid reagent
- Alpha napthylamine reagent

**6. Voges-Proskauer reagent (Oxoid):**

5% alpha-naphthol in absolute alcohol and 40% potassium hydroxide solution were used for the Voges-Proskauer test.

### 3.1.4. Stains (Mackie and MacCartney, 1996):

#### 3.1.4.1. Gram's stain:

It was used to differentiate the isolated organisms into Gram positive and Gram negative.

#### 3.1.4.2. Leishman's stain:

It was used to detect *P. multocida* bipolarity in blood smears from chickens and mice.

### 3.1.5. *P. multocida* specific antisera:

The specific A and D capsular antisera and somatic antisera 1,3 and 4 were obtained from the National Animal Disease, Central Laboratories, Ames, Iowa, USA, and were used for serological typing of *P. multocida*.

### 3.1.6. Materials used for serological identification of *P. multocida*:

#### 3.1.6.1. Materials used for indirect haemagglutination (IHA) test:

1. Sheep RBCs.

2. 40% formaldehyde solution.

3. 96 U-shaped microplate.

4. Multichannel and unichannel micropipettes.

5. Physiological saline 0.85%.

6. 500ml capacity flasks.

7. Dialyzing tube.

8. Gauze

9. Shaker.

**3.1.6.2. Materials used for agar gel precipitation test:**

1. Agarose 1% **(Difco)**.

2. Physiological saline 0.85%.

3. Thiomersal 0.01%.

4. 25 X 75 mm glass slide.

5. 0.3 ml 40% Formalin.

6. Water bath.

**3.1.7. Materials used for molecular identification of *P. multocida* isolates using conventional polymerase chain reaction (cPCR):**

**3.1.7.1. Material used for extraction of DNA**

**3.1.7.1.1. QIAamp DNA Mini Kit** Catalogue no.**51304**

The QIAamp DNA Mini Kit provides silica-membrane-based nucleic acid purification from different types of samples. The spin-column procedure does not require mechanical homogenization, so total hands-on preparation time is only 20 minutes.

**3.1.7.1.2. Ethanol 96% (Applichem)**

### 3.1.7.2. Equipment and apparatuses used for extraction of nucleic acids:

A. Epindorff tubes 1.5 ml capacity.

B. Monochannel micropipettes 20-200 µl, 100-1000 µl **(Biohit)**.

C. Sterile filter tips (200 µl, 1000µl) capacity.

D. Centrifuge **(Sigma Sartorius)**.

E. Type II-A bio safety cabinet **(Thermo).**

### 3.1.7.3. PCR Master Mix used for cPCR:

**Emerald Amp GT PCR mastermix (Takara)** Code No. **RR310A**

Contains:

1) Emerald Amp GT PCR master mix (2x premix).

2) PCR grade water.

### 3.1.7.4. Oligonucleotide primers used in cPCR Metabion (Germany)

They have specific sequence and amplify a specific product as shown in **table 6.**

**Table (6): Oligonucleotide primers sequences (OIE, 2012)**

| Target gene | Primers sequences | Amplified Segment (Bp) |
|---|---|---|
| **KMT1** | ATC-CGC-TAT-TTA-CCC-AGT-GG | 460 |
| | GCT-GTA-AAC-GAA-CTC-GCC-AC | |
| **Serogroup A** | TGC- CAA- AAT-CGC-AGT-GAG | 1044 |
| | TTG-CCA-TCA-TTG-TCA-GTG | |

Bp= Base pair

**3.1.7.5. DNA molecular weight marker:**

**3.1.7.5.1. Gel Pilot 100 bp ladder (**Cat. No. 239035) supplied fromQIAGEN **(USA).**

Number of bands: 6

Size range: 100-600 bp.

**3.1.7.5.2. Gel Pilot 100 bp plus ladder** (cat. no. 239045)

It was supplied from QIAGEN **(USA).**

Number of bands: 11

Size range: 100-1500 bp.

**3.1.7.6. Material used for agarose gel electrophoresis:**

**3.1.7.6.1. Agarose 1% (Sambrook et al., 1989)**

A multi-purpose, high gel strength agarose suitable for a wide range of molecular biology techniques was used. As it has high gel strength and exclusion limits, multi agarose could effectively separate large DNA fragments with reduced running times. This in turn means less band diffusion, a problem often associated with long running time. It was prepared as follow:

Agarose powder (ABgene) 1 g

TBE 100 ml

**3.1.7.6.2. Ethidium bromide solution 10 mg / ml (Sambrook et al., 1989):**

Ethidium bromide powder (Sigma) 10 mg

Sterile distilled water 1.0 ml

It was mixed and stored covered at 4°C

It was added to melted agarose to reach a final concentration of 0.1-0.5 µg /ml.

**3.1.7.6.3. Tris borate EDTA (TBE) electrophoresis buffer (1x) (WHO, 2002):**

| | | |
|---|---|---|
| Tris buffer | (Fluka) | 10.87 g |
| Boric acid | (Fluka) | 5.5 g |
| EDTAdiNA | (Winlab) | 0.82 g |

It was brought up to 1 liter with deionized water, pH was checked up. If the pH was out of the range of 8-8.6, and a new solution was prepared again. Any change in ion concentration would affect the migration of the DNA through the gel.

### 3.1.7.7. Equipment and apparatuses used in cPCR:

a. Calibrated cylinders.
b. Glass flasks.
c. PCR tubes 0.2 ml capacity.
d. Balance **(Scaltec)**.
e. Microwave **(Panasonic)**.
f. Monochannel micropipette (2-20 µl) **(Biohit)**.
g. Sterile filter tips.
h. Gel casting apparatus **(Biometra).**
i. T3 Thermal cycler **(Biometra)**.
j. Power supply **(Biometra)**.
k. Type II A biosafety cabinet **(Thermo)**.
l. Gel documentation system **(Alpha Innotech)**.
m. Deionizer **(Millipore)**.
n. Double distillatory **(Sanyo)**.

### 3.1.8. Material used for preparation of inactivated *P. multocida* vaccine:

#### 3.1.8.1. Formalin working solution (ADWIA):

40% Formalin was used for bacterial inactivation at 0.2% final concentration.

#### 3.1.8.2. Aluminium hydroxide gel (2%) (Merck):

It was used in a concentration of 20% as adjuvant added to inactivated vaccine.

### 3.1.9. Experimental animals and birds:

#### 3.1.9.1. Mice:

A total of 250, Swiss Albino mice about 15-20 gm was supplied by Central Laboratory for Evaluation of Veterinary Biologics, Abbasia, Cairo, Egypt were used for pathogenicity test and evaluation of lethal dose 50 ($LD_{50}$) of the isolated *P. multocida* strains.

A total of 25 Swiss white mice about 18-20 gm body weight were used for evaluation of *P. multocida* vaccine safety.

#### 3.1.9.2. Chickens:

A- The experiment was done according to the National Regulations of Animal Welfare and Institutional Animal Ethical Committee (IAEC). A total of 200, day-old layer chickens was obtained from local hatcheries and reared on thoroughly cleaned and disinfected semi-closed houses for 13 weeks. Birds were vaccinated using standard protocol for vaccination. All chicks were vaccinated against Newcastle disease (ND) virus at 7 and 21 day of age by using live Hitchner B1 and La Sota strains vaccines; respectively. Live infectious bursal disease (IBD) vaccine was administrated at 14 days of age. Drinking water method was used as route of administration of previously mentioned live vaccines. On the day 10 of age, 0.5 ml inactivated avian influenza (AI) vaccine (H5N1) was inoculated

subcutaneously at the back of the bird's neck. Birds were fed on commercial balanced ration without antibiotics. Feed and water was given *adlibitum*. Semduramicin was added at a concentration of 25 ppm as a coccidiostat, during the experimental period.

B- Ten chickens were also used for testing the safety of the prepared vaccine.

### 3.1.10. Bacterial strain used for challenge:

The local isolates of *P. multocida* of serotype A:1 and A:3 isolated from layers and breeders type chickens were used as challenge bacteria in the challenge test.

### 3.1.11. Materials and equipment used for enzyme linked immunosorbent assay (ELISA) test:

The commercial *P. multocida* ELISA test kits (IDEXX Laboratories, Inc) were used to measure the relative level of antibody to *P. multocida* in chicken serum.

#### 3.1.11.1. Reagents:

- *P. multocida* antigen coated plates.
- Positive control:

Diluted chicken anti- *P. multocida* serum was preserved with sodium azide.

- Negative control:

Diluted chicken serum non-reactive to *P. multocida* was preserved with sodium azide

- Conjugate :

Goat anti chicken: HRPO conjugate, preserved with gentamycin and Kathon.

- Sample diluents:

Buffer was preserved with sodium azide.

- TMB substrate
- Stop solution

### 3.1.11.2. Equipment:

- 96-well microplate reader **(Versa Max).**
- Precision micropipettes and multi-dispensing micropipettes **(Biohit).**
- Disposable pipette tips.
- Micro-plate washer.
- Vortex or equivalent **(Bionex)**.

### 3.1.12. Materials used for histopathological examination:

- 10% neutral formalin.
- Ethyle alcohol.
- Methyl alcohol.
- Absolute ethyle alcohol.
- Xylol.
- Paraffin wax.
- Hematoxylin and Eosin (H&E) stain.

### 3.1.13. Antibiogram:

Two isolated strains of *P. multocida* (A:1 and A:3) were tested *in-vitro* for their susceptibility to 13 antimicrobial agents **(Oxoid)** using the disc diffusion technique. The used discs were the followings; norfloxacin (NOR, 10 µg), gentamycin (CN, 10 µg), tetracycline (TE, 30 µg), erythromycin (E, 15 µg), streptomycin (10 µg), trimethoprim/sulphamethoxazole (SXT, 1.25/23.75 µg), cefoperazone (CEP, 75 µg), ampicillin (AM,10µg), ofloxacin (OFX, 5 µg), chloramphenicol (C, 30 µg), penicillin G (P, 10 Iµ),azithromycin (AZM, 15 µg) and clindamycin (DA, 2 µg).

# 3.2. METHODS

## 3.2.1. Isolation and morphological identification of *P. multocida*:

Samples from liver, heart and spleen from each bird were inoculated under aseptic condition into brain heart infusion broth and then incubated at 37°C for 18-24 hr **(Cowan, 1985 and Bhimani et al., 2014).**

Inoculated broth was further cultured onto DAS medium, blood agar and MacConkey agar and then incubated under aerobic conditions for 18-24 hr at 37°C. The agar plates were checked every day for suspected *P. multocida* colonies **(Holt et al., 1994, Sthitmatee et al., 2008 and Mohamed et al., 2012).**

Suspected *P. multocida* colonies were examined for their colonial morphology. Films were prepared from the suspected pure *P. multocida* colonies and stained with Gram's stain. Blood films were prepared from blood of living chickens and liver of dead ones and stained with Leishman's stainfor detection of specific bipolarity **(Cruickshank et al., 1975 and Cheesbrough, 2006).**

## 3.2.2. Biochemical identification of suspected *P. multocida* isolates: (Quinn et al., 2002 and Kumar et al., 2012)

The suspected *P. multocida* colonies were subjected to the following biochemical tests:

### 3.2.2.1. Oxidase test:

The colonies were picked by loopfull and touched on the disc. The observed blue color was considered as positive result.

### 3.2.2.2. Catalase test:

2-3 drops of 3% $H_2O_2$ was mixed with bacterial colony on clean slide. The presence of gas bubbles was considered as positive result.

### 3.2.2.3. Indole test:

0.5 ml of Kovac's reagent was added into the tube containing 24 hr bacterial culture. Formation of red ring indicated positive result whereas yellow ring indicated negative result.

**3.2.2.4. Urea hydrolysis test:**

The urea agar medium was inoculated with a loopful of pure culture and incubated at 37$^{o}$C for 24-48 hr. Urease positive cultures produced purple color due to splitting of urea with production of ammonia on phenol red indicator whereas yellow color indicated negative reaction.

**3.2.2.5. Triple sugar iron:**

It was used for detection of fermentation of glucose, lactose, and sucrose and $H_2S$ production after inoculation at 37$^{o}$C for 24 hr.

**3.2.2.6. Nitrate reduction test:**

Inoculum of tested organism was incubated in nitrate broth and incubated at 37$^{o}$C for 48 hr. Reduction of nitrate to nitrite was detected by adding 1 ml of sulfanilic acid reagent and 1 ml of α-naphthylamine reagent to nitrate broth culture. Development of a distinct red colour was considered as positive test.

**3.2.2.7. Voges-Proskauer test:**

One drop of alpha-naphthol and potassium hydroxide was added to 24 hr old culture of Voges-Proskauer broth to determine the ability of formation of acetyl methyl carbinol. Red colour indicated a positive result whereas a yellow colour indicated a negative result.

**3.2.2.8. Sugar fermentation test:**

Peptone water base with 1% Andrade's indicator was added to 1% of the following sugars; glucose, sucrose, lactose, maltose mannitol, dulcitol, xylose, arabinose and fructose.

**3.2.3. Mice inoculation:**

For detection of pahogenicity of *P. multocida* strains, identified strains were incubated in brain heart infusion broth for 24 hrs. Six mice for each isolate were inoculated I/P with 0.2 ml containing $2.4 \times 10^8$ CFU/ml. Inoculated mice were observed for 3 days for mortalities. From dead mice, heart blood was collected and streaked on blood agar and liver impression smear was taken and stained by Leishman's for microscopical examination of *P. multocida* organism **(Shivachandra et al., 2005 and Mohamed et al., 2012).**

### 3.2.4. Serological identification of *P. multocida* isolates:

#### 3.2.4.1. Capsular typing by (IHA) test: (Carter and Rappy, 1962)

#### A. Preparation of formalized erythrocytes:

Fresh defibrinated sheep blood was washed 5 times with 0.85% cold normal saline. The red cells were packed after final centrifugation at 2300 rpm for 15 min, then resuspended in 8 volumes of saline and placed in a 500 ml capacity conical flask. A quantity of 40% formaldehyde solution pH 5.5-6.0 was introduced into dialyzing tube which immersed into the sheep erythrocytes flask. The flask was then shaked for 2 hr at room temperature. After agitation, the content of dialysis tube was poured into the flask and shaking was continued for additional 16-18 hr. The suspension was filtered through gauze. The filtered cells were washed 6 times in saline. The packed cells were suspended in sufficient quantity of saline solution to make 50% suspension and preserved for use at 4°C.

#### B. Preparation of *P. multocida* capsular extract:

An 18-24 hr confluent *P. multocida* growth from blood agar plates was washed off with 4.5 ml saline and heated at 56°C for 30 min. The bacterial cells were separated by centrifugation at 1500 rpm for 10 min and the supernatant fluid which contains the capsular antigens was transferred to another tube.

#### C. Sensitization of formalized erythrocytes with capsular extract:

0.2 ml of packed erythrocytes was added to 3 ml bacterial extract which were then thoroughly mixed and incubated at 37°C for 2 hr. The red cells were separated by centrifugation at 1500 rpm for 15 min and washed once with 10ml of saline after which sufficient saline was added to give 1% final concentration.

**D. Performance of IHA test:**

Two fold dilutions of the *P. multocida* specific antisera were prepared in the wells of a multiple U-shaped plates. Then the sensitized red cells were added in each well. The plates were vigorously shaked and the results were recorded after 2 hr in room temperature. A second reading was recorded after 24 hr in the refrigerator.

Positive reactions consisted of marked diffuse haemagglutination, whereas a negative pattern consisted of compact sharply demonstrated disc of sedimented cells (button like).

**3.2.4.2. Somatic typing using gel diffusion precipitation test: (Heddleston et al., 1972)a**

**A. Preparation of the somatic antigen:**

An 18-24 hr heavy seeded growth culture of *P. multocida* was suspended in 1ml of 0.85% NaCl solution containing 0.3% saturated solution of formaldehyde. The suspension of cells was heated in a water bath at 100°C for 1 hr. The cells were sedimented by centrifugation, and the supernatant was as (O) antigen in the gel diffusion precipitation test.

**B.Agar gel diffusion precipitation test: (Hofacre and Glisson, 1986).**

The agar gel consisted of 0.9% special agarose, 8.5% NaCl, and 0.01% thimerosal in distilled water. Five ml of melted agar was placed on microscopic slides, and 5 or 7 wells, 4 mm in diameter were cut. Antisera were placed in the outer wells and the antigens in center wells. The slides were kept in a moist chamber at 37°C. The results were recorded after 24, 48 and 72 hr. The specific

line of precipitation (heat stable) was close to the top of antigen wells while the non-specific line (heat labile) was close to the serum wells.

### 3.2.5. Molecular identification of *P. multocida* isolates by cPCR:

#### 3.2.5.1. Extraction of DNA:

According to **QIAamp DNA mini kit instructions**

1- 20 µl QIAGEN protease were pipetted into the bottom of a 1.5 ml microcentrifuge tube.

2- 200 µl of the culture broth of isolates were added.

3- 200 µl buffer AL were added to the sample, mixed by pulse vortexing for 15 seconds.

4- The mixture was incubated at 56°C for 10 min.

5- The 1.5 ml microcentrifuge tube was centrifugated to remove drops from the inside of the lid.

6- 200 µl ethanol (96%) were added to the sample, and mixed again by pulse vortexing for 15 seconds. After mixing, the 1.5 ml microcentrifuge tube was briefly centrifugated to remove drops from the inside of the lid.

7- The mixture from step 6 was carefully applied to the QIAamp mini spin column (in a 2ml collecting tube) without wetting the rim. the cap was closed, and centrifugated at 8000 rpm for 1 min. The QIAamp mini spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded.

8- The QIAamp mini spin column was carefully opened and 500 ml buffers AW1 were added without wetting the rim. The cap was closed, and centrifugated at 8000 rpm for 1 min. The QIAamp mini spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded.

9- The QIAamp mini spin column was carefully opened and 500 ml buffers AW2 were added without wetting the rim. The cap was closed, and centrifugated at full speed for 3 min.

10- The QIAamp mini spin column was placed in a new 2 ml collection tube and the old collection tube was discarded with the filtrate. Centrifugation at full speed for 1 min was done.

11- The QIAamp mini spin column was placed in a clean 1.5 ml microcentrifuge tube, and the collection tube containing the filtrate was discarded. The QIAamp mini spin column was carefully opened and 100 µl buffer AE were added. The QIAamp mini spin column was incubated at room temperature (15-25ºC) for 1 min, and then centrifugated at 8000 rpm for 1 min.

**3.2.5.2. Preparation of PCR Master Mix:** [Emerald Amp GT PCR Master Mix (Takara) Code No. RR310A kit]as shown in **(Table 7).**

**Table (7): Preparation of uniplex PCR Master Mix**

| Component | Volume / reaction |
|---|---|
| Emerald Amp GT PCR Master Mix (2x premix) | 12.5 µl |
| PCR grade water | 4.5 µl |
| Forward primer (20 pmol) | 1 µl |
| Reverse primer (20 pmol) | 1 µl |
| Template DNA | 6 µl |
| Total | 25 µl |

**3.2.5.3. Cycling conditions of the primers during cPCR:**

Cycling (temperature and time) conditions of the primers during PCR are shown in **table 8** according to **OIE (2012)** and **Emerald Amp GT PCR master mix (Takara) kit.**

**Table (8): Cycling conditions of the different primers during cPCR**

| | Amplification (35 cycles) | Final |
|---|---|---|

| Primary denaturation | Secondary denaturation | Annealing | Extension | extension |
|---|---|---|---|---|
| 94°C<br>5 min. | 94°C<br>30 sec. | 55°C<br>1 min. | 72°C<br>1 min. | 72°C<br>10 min. |

**3.2.5.4. DNA molecular weight marker**:

The ladder was mixed gently by pipetting up and down. 6 µl of the required ladder were directly loaded.

**3.2.5.5. Agarose gel electrophoreses: (Sambrook et al., 1989)** with modification:

Electrophoresis grade agarose (1g) was prepared in 100 ml TBE buffer in a sterile flask, it was heated in microwave to dissolve all granules with agitation , and allowed to cool at 70°C, then 0.5 µg / ml ethedium bromide was added and mixed thorough.

The warm agarose was poured directly in gel casting apparatus with desired comb in apposition and left at room temperature for polymerization.

The comb was then removed, and the electrophoresis tank was filled with TBE buffer. Twenty µl of each uniplex PCR product samples, and negative control and positive control were loaded to the gel. The power supply was 1-5 volts/cm of the tank length. The run was stopped after about 30 min and the gel was transferred to UV cabinet.

The gel was photographed by a gel documentation system and the data was analyzed through computer software.

**3.2.6. Preparation of inactivated vaccine from the predominant *P. multocida* local strains:**

A bivalent vaccine was prepared from the *P. multocida* strains (A:1 and A:3) according to method described by **Borkowska-Opacka et al. (1996)**. Simply, *P. multocida* strains were grown in brain heart infusion broth at 37ºC for 16-24 hr. Formalin was added to the culture in final concentration of 0.2% and the formalized culture was re-incubated at 37ºC for 24 hr. Aluminium hydroxide gel 2% was added at concentration of 20% and was mixed well with the culture. The vaccine was standardized to contain $10^8$ CFU/0.5ml dose.

### 3.2.7. Quality control tests of the locally prepared inactivated *P. multocida* vaccine:

The prepared vaccine was tested for sterility, safety and potency according to **British Veterinary Codex (1970) and Code of American Federal Regulation (1985).**

#### 3.2.7.1. Sterility test:

For confirmation that the prepared vaccine was free from any bacterial and fungal contamination, one ml from the vaccine was inoculated into each of the three vessels of FTM, FTMB and SCDM of volume not less than 10 ml.

Test vessels of FTM and FTMB were incubated at 30-35ºC and test vessels of SCDM were incubated at 20-25ºC for not less than 14 days. At intervals during the incubation period, the test media were examined for macroscopic evidence of microbial growth.

#### 3.2.7.2. Safety test:

This test was carried out by inoculation of ten chickens with double dose of prepared *P. multocida* inactivated vaccine. All chickens were kept under observation for 14 days for any abnormal reactions.

About 0.2ml of the prepared *P. multocida* vaccine were inoculated in Swiss white mice to confirm the safety test.

#### 3.2.7.3. Potency test:

It was done by vaccination and challenge.

**A. Vaccination of chickens by the locally prepared inactivated *P. multocida* vaccine:**

Primary vaccination was done at the age of 6 weeks, 0.5 ml of the prepared inactivated *P. multocida* vaccine containing $10^8$ CFU/0.5 ml was inoculated I/M into the thigh region. Booster vaccination was done at 9 weeks of age (3 weeks after primary vaccination) **(Borkowska-Opacka et al., 1996)**.

**B. Challenge test against *P. multocida*:**

Both vaccinated and non-vaccinated control chickens were challenged with *P. multocida* serotypes A:1 and A:3, 2 weeks after booster vaccination in a dose of 0.1 ml of bacterial suspension containing $10^7$/ml that inoculated I/M. Birds were observed for 14 days later **(Sthitmate et al.,2008)**.

**3.2.8. Experimental design:**

Experiments were carried out on a total of 200, 6 weeks old chickens reared under hygienic condition and divided into 5 equal groups as followed:

**Group (1):** Forty chickens were vaccinated with 0.5 ml of prepared *P. multocida* vaccine I/M and booster dose was given after 3 weeks. All birds were challenged with virulent *P. multocida* serptype A:1, 2 weeks after the booster vaccination.

**Group (2):** Forty chickens were vaccinated with 0.5 ml of prepared *P. multocida* vaccine I/M and booster dose was given after 3 weeks. All birds were challenged with virulent *P. multocida* serptype A:3, 2 weeks after the booster vaccination.

**Group (3):** Non-vaccinatedforty chickens were challenged with *P. multocida* serotype A:1 (positive control).

**Group (4):** Non-vaccinated forty chickens were challenged with *P. multocida* serotype A:3 (positive control)**.**

**Group (5):** Non-vaccinated and non-challenged forty chickens wereleft as (negative control).

The experimental design is shown in **(Table 9).**

**Table (9): The experimental design of vaccinated and challenged groups with *P. multocida***

| Group | Vaccination | Challenge |
|---|---|---|
| **1** | + | + (A:1) |
| **2** | + | + (A:3) |
| **3** | - | + (A:1) |
| **4** | - | + (A:3) |
| **5** | - | - |

### 3.2.9. Parameters used for evaluation of locally prepared inactivated *P. multocida* vaccine in chickens:

#### 3.2.9.1. Immunological parameters:

Detection of humeral immune response of vaccinated and challenged birds were done using ELISA test. Blood samples were collected from the wing vein of birds in each group pre-vaccination and weekly after vaccination till the end of the study to determine the serum antibody titer of chickens.

**Procedure of ELISA test:**

It was used for estimation of antibody titers in chicken sera that vaccinated with locally prepared inactivated *P. multocida* vaccine using *P. multocida* antibody test Kit (IDEXX). The test was performed according to the description of manufacture instructions as follows:

**1. Serum dilution:**

Serum samples were diluted as 1:500 with sample diluents.

**2. Addition of positive control:**

100 µl of positive control were added into duplicate wells.

**3. Addition of negative control:**

100 µl of negative control were added into duplicate wells.

**4. Addition of serum samples:**

100 µl of diluted sample were added into appropriate wells. The plate was incubated at 18-26ºC for 30 min.

**5. Addition of conjugate:**

The plate contents were removed and washed with distilled or deionized water 3-5 times then 100 µl of conjugate were added into each well and then incubated at 18-26ºC for 30 min.

**6. Addition of substrate:**

The plate contents were removed and washed with distilled or deionized water 3-5 times then 100 µl of TMB substrate were added into each well and the plate was kept at 18-26ºC for 15 min.

**7. Addition of stopping solution:**

The reaction was stopped by adding 100 µl of stop solution and the plate was read using ELISA reader at 650 nm.

**8. Calculation of antibody titers:**

The results of antibody titers were determined from S/P ratio:

$$\text{S/P: } \frac{\text{Sample mean – negative control}}{\text{positive control} - \text{negative control}}$$

$$\text{Log}_{10}\ \text{Titer} = 1.09\ (\log_{10}\ \text{S/P}) + 3.36$$

**3.2.9.2. Clinical parameters**

The clinical signs, mortality rate and post-mortem lesions which are specific for *P. multocida* were recorded in groups post challenge for measuring vaccine protection rate.

**3.2.9.3. Re-isolation rate:**

Liver was collected from dead as well as sacrificed living birds at the end of the study in each group for *P. multocida* re-isolation. It was done by inoculation

of the liver on blood agar media and then by morphological and biochemical identifications.

### 3.2.9. 4. Histopathological examination:

Three chickens from each group were sacrificed at the end of study (13 weeks old) for histopathological examination and tissue specimens were collected from heart, liver and spleen and then fixed in 10% formol saline solution.

After proper fixation, the specimens were dehydrated in ascending grades of ethyl alcohol, serially diluted in alcohol (methyl, ethyl and absolute ethyl), cleared in xylol and embedded in paraffin at 56$^{o}$C in hot air oven for 24 hr.

Thin tissue sections about 5 microns in thickness were prepared and stained with H&E stain for histopathological examination with the light microscope (**Bancroft and Gamble, 2007)**.

### 3.2.10. Antibiogram:

Four to five colonies of *P. multocida* were picked and suspended in sterile saline, the turbidity of organism growth was adjusted to 0.5 Mcfarland standard tube. Sterile cotton swab was dipped into the prepared inoculum tube and spread uniformly into Muller Hinton agar, then antibiotic discs were dispensed on the surface of the agar using forceps and the plates were incubated at 37$^{o}$C for 24 hr. The zones of inhibition were measured and recorded to determine the sensitivity or resistance of *P. multocida* to the tested drugs according to the standardized protocol that was recommended by **Clinical Laboratory Standards Institute (CLSI) (2017)**.