5. DISCUSSION

Fowl cholera caused by *P. multocida* is a bacterial disease affecting domestic and wild birds inducing devastating economic hazards **(Christensen and Bisgaard, 2000, Aye et al., 2001, Pedersen et al., 2003, Biswas et al., 2005, Chrzastek et al., 2012 and Singh et al., 2014)**. All ages are susceptible to *P. multocida* infection; however, death losses of chickens usually occur in laying flocks **(Heddleston and Watko, 1965, Choudhury et al., 1985, Rhoades and Rimler, 1991 and Muhairwa et al., 2001)**.

Diagnosis of FC depends on identification of the causative agent, following isolation from suspected birds showing signs and lesions consistent with this disease **(Anonymous, 2000)**.

In this study, a total of 55 layers and breeders chicken flocks with clinical signs and post-mortem lesions suggestive to FC infection were investigated for the presence of *P. multocida* from different localities at El-Sharqia, El-Minofia, El-Qalubia and El-Gharbia governorates, Egypt. Out of which 6 isolates were confirmed to be *P. multocida* with total incidence rate of 10.9%. Five out of 39 and one out of 16 samples were positive *P. multocida* with incidence rates of 12.8 % and 6.2 % for layers and breeders; respectively. The incidence rate of *P. multocida* was the highest in El-Sharqia with total percentage of 13.6% (13.3% for layers and 14.28 % for breeders), followed by El-Minofia governorate with total percentage of 12.5% (16.67 % for layers and 0 % for breeders), El-Qalubia with total percentage of 9% (12.5 % for layers and 0 % for breeders) and finally El-Gharbia with total percentage of 7.1% (10 % for layers and 0 % for breeders

The data of this study agree with those reported by some Egyptian researchers like **Hussin (1988)** who isolated 31 *P. multocida* out of 359 examined cases from layers in Sharkia province with incidence of 8.6%. **Abd El-Dayem (1990)** who isolated *P. multocida* from 45 out of 500 samples (9%) from Kaloubia

governorate. Moreover, **Mohamed et al. (2012)** reported that out of 275 examined backyard chickens samples collected from different regions of Upper Egypt, 21 (7.6%) isolates of *P. multocida* were recovered and confirmed by using phenotypic characterization. In addition, **El-Shamy (2008)** recorded that out of 128 diseased and dead birds represented 21 farms suspected to be infected with FC, 14 *P. multocida* strains were isolated with percentage of 13.81; the highest percentage of isolation of the organism was in El-Sharkia governorate (8.57%) followed by El-Garbia governorate (2.94%), and then EI- Behera governorate (2.3%). **Mona (2015)** isolated 8 strains of *P. multocida* (6.25%) from layers in different Egyptian governorates.

Similarly, **Arshed et al. (2003)** isolated *P. multocida* from 16 layer flocks aged 18-24 weeks in and around Faisalabad. **Mehmood et al. (2016)** confirmed presence of different *P. multocida* serotype A isolates in 5 commercial layers flocks of Karachi, Lahore, Vehari and Toba Tek Singh (Pakistan). In india, **Pillai et al. (2013)** recovered 10 isolates of *P. multocida* serotype A:1 from 155 samples of poultry and deer. In Bangladesh, several studies were done on the prevalence of FC where **Hasan et al. (2010**), **Belal (2013) and Hossain et al. (2013)** found the disease incidence 12.72% in layers and 4.25% in broilers, 59.72% in backyard poultry and 13.04% in chicken; respectively. Moreover **Akhtar et al. (2016)** detected conventionally and molecularly 2 isolates of *P. multocida* out of 5 suspected dead chickens. **Balakrishnan and Parimal (2012)** identified *P. multocida* isolates from chickens and turkeys, in Chennai, on the basis of biochemical characteristics, pathogenicity studies in mice and PCR.

The differences in isolation rates may be due to the number of samples, method of isolation, presence of stress and age of birds that were examined.

In this work, the isolation of *P. multocida* from liver, heart and spleen on specific media revealed presence of grayish and small mucoid dew drop like colonies on DSA and brain heart infusion agar, non haemolytic colonies on blood

agar after 48 hr. incubation at 37ºC and there was no growth on MacConkey agar. As well, *P. multocida* has been detected in liver, spleen, blood and lungs of infected birds **(Hunter and Wobeser, 1980)**. For isolation of*P.multocida*, selective enrichment along with inoculation of blood agar increases the isolation rate **(Moore et al., 1994)**. **Mohamed et al. (2012)** isolated *P. multocida* from brain heart infusion broth after incubation at 37ºCfor 18-24 hr. and then subculturing on sheep blood agar.

The colonies characteristics of *P. multocida* organisms are in support with the findings of **Divivedi and Sodhi (1989), Shivachandra et al. (2005) and Christensen and Bisgaard (2006)**.

Gram stained smears from suspected *P. multocida* colonies showed Gram negative coccobacilli. Blood films and liver impression smears taken from septicaemic diseased and dead chickens suspected to be infected with *P. multocida* showed bipolar organisms after staining with Leishman's stain. These findings are accordance with **Purushothman et al. (2008); Ashtaf et al. (2011); Sarangi and Panda (2011) and Mehmood et al. (2016)**.

All suspected *P. multocida* isolates were positive to catalase, oxidase, indole production, hydrogen sulphide production, nitrate reduction test, Voges-Proskauer test and sugar fermentation reactions of glucose, sucrose, fructose, mannitol and xylose while were negative for urease activity, sugar fermentation reactions of maltose, lactose, arabinose and dulcitol. These findings of biochemical reactions for *P. multocida* are parallel with the findings of **Patel (2004) and Zhang et al. (2004)**.

Mouse assay is an efficient and widely accepted method for detection of *P. multocida* **(Baladrias et al., 1988)**. The isolated *P. multocida* strains were pathogenic for mice, where mice died within 18-24 hr after experimental infection, congestion and septicaemia of the internal organs. Leishman's stained

liver impression smears revealed bipolarity. These results are in agreement with the findings of **Balakrishnan and Mini (2001)**.

This study showed that *P. multocida* isolates were confirmed serologically to be (A) capsular type using specific capsular antisera and (1) and (3) somatic types using specific antisera. Indirect haemagglutination and agar gel diffusion tests were carried out and *P. multocida* isolates were confirmed as A:1 and A:3 serotypes. In the same manner, **Shivachandra et al. (2006) and Glisson et al. (2008)** demonstrated that capsular serotypes of *P. multocida* including A, B, D, E and F were found in chickens, as serotype A caused FC in avian species. **Mohamed et al. (2012)** demonstrated that *P. multocida* serotype A:1 strains caused 80% mortality, however, type D strains caused 20% mortality in chickens in Egypt. Also, **Shimaa (2016)** isolated 14 *P. multocida* strains and the serological identification revealed that they were serotypes A:1 and A:4, and **Hassan et al. (2001)** isolated *P. multocida* type D and A: 3,4 from laying chickens flocks.

Cultivation and identification of *P. multocida* by standard bacteriological methods can be ambiguous because V-factor requirement or non-typeable strains including cross-reactions in serotyping and viable but non-culturable cells **(Krause et al., 1987)**. **Townsend et al. (2001)** indicated that the detection of *P. multocida* from direct culture was less efficient than either mouse inoculation or PCR, as well as detection of *P. multocida* by PCR was particularly successful as some isolates were shown to be non pathogenic for mice. PCR technique is used usefully for detection of DNA of *P. multocida* **(Townsend et al., 1998 and Purushothaman et al., 2008).**

In this work, molecular detection of *P. multocida* by specific PCR (PM-PCR) was used to identify *P. multocida* isolates by amplifying the KMT1 gene. The primer amplified product of 460 bp, and the molecular serotyping of the isolated *P. multocida* strains using serotype (A) specific primer revealed that the

isolated strains belonged to serogroup (A) at 1044 bp. Parallel results was reported by **Ranjan et al. (2011) and Akhtar (2013)**. In Bangladesh, **Panna et al. (2015)** confirmed presence of *Pasteurella* species type A in chickens by PCR. **Zhang et al. (2004)** investigated that most *P. multocida* isolates were serotype A:1 (57.14%) which was considered the main cause of acute form of FC. Ninety two out of 123 *P. multocida* strains belonged to serotype A:1 was detected by **Shivachandra et al. (2006)**. In Asian countries, common serotype of *P. multocida* which associated with FC was A:1 after characterization with PCR **(Gunawardana et al., 2000, Townsend et al., 2001, Kumar et al., 2004 and Dziva et al., 2008)**.

From the above mentioned results, it could be concluded that *P. multocida* strains are circulating in Egyptian layers and breeders chickens flocks despite of vaccination and medication. So, continuous surveillance studies are important to detect the presence of the new strains of *P. multocida* in the field.

Fowl cholera is one of the most important problems facing poultry industry, so vaccination against the disease is practiced as preventive measures in many countries of the world including Egypt **(Gergis, 1978)**. Both live and inactivated *P. multocida* vaccines (bacterin) have been attempted to prevent the disease **(Glisson et al., 2008)**. Inactivated *P. multocida* vaccines are widely used as the organisms do not have chance to be reverted to virulence to cause the disease **(Hopkins and Olson, 1997)**.

In the present work, a local bacterin was prepared using *P. multocida* field strains and its efficacy was determined. The results showed that the locally prepared inactivated *P. multocida* vaccine was free from any bacterial or fungal contaminations and it was safe and produced no clinical signs among the vaccinated chickens during the observation days. As well as it was safe for inoculated mice.

The potency of the locally prepared *P. multocida* bacterin was measured by using ELISA test to determine the humoral immune response in different chicken groups. In both groups (1) (vaccinated and challenged birds with *P. multocida* type A1) and (2) (vaccinated and challenged birds with *P. multocida* type A3), the mean ELISA antibody titers increased from (80) pre-vaccination level to reached 2260 and 2010 at the 3th week after primary vaccination; respectively, however, two weeks after secondary vaccination, the antibody titers reached 4350 and 3980 respectively, then declined to 2998 and 2679 one week after challenge then increased to 3970 and 3905 at two weeks after challenge, respectively. Results of the mean ELISA antibody levels in controls groups (3) (non vaccinated and challenged birds with *P. multocida* type A:1) and (4) (non vaccinated and challenged birds with *P. multocida* type A:3) were 60-80 before challenge and then increased to 95 at two weeks after challenge. Group 5 (non vaccinated-non challenged control) showed steady mean ELISA antibody levels (65-80).

The antibody titers measured by ELISA was at least twice as sensitive as IHA **(Solano et al., 1983)**. Furthermore, **Avakian et al. (1989)**, **Perelman et al. (1990) and Esmaily et al. (2003)** recorded that polyvalent FC oil-based bacterin induced high antibody titer in broiler mini breeder hens measured by ELISA technique. In addition, **Jabbri and MoazeniJula (2005)** stated that inactivated trivalent FC vaccine consisted of serotypes 1, 3 and 4 *P. multocida* strains induced immunogenic response in vaccinated chickens, as ELISA assay showed a considerable increase in antibody titer after twice vaccination of 8 weeks old chickens. **Muhammad (2005)** used ELISA assay to detect the antibodies in vaccinated chickens with CU FC vaccine, where birds vaccinated two or three times between the ages of 7 and 20 weeks became sufficiently immune and tolerated FC challenge, while those not vaccinated or only vaccinated at the age of 7 weeks were not sufficiently immunized.

The results of humoral immune response that obtained in this study were comparable to that obtained by **Akhtar et al. (2016)** who prepared a formalin killed FC vaccine and determined the antibody titer in chickens aging 15 weeks either by injecting 1 ml (group A) or o.5 ml (group B). It was found that antibody titers in the birds of group A and group B were 4.513 and 4.07; respectively after primary vaccination, and 4.843 and 4.37; respectively after booster vaccination.

The good immune response of the birds to *P. multocida* bacterin was explained by **Harper et al. (2012 and 2016)** who reported that the capsule and lipopolysaccharide (LPS) of *P. multocida* constitute the major components of the bacterial cell surface. They are primary stimulators of the host immune response and critical determinants of bacterin protective efficacy. They play key roles in a range of interaction between the bacteria and the hosts they colonize or infect. Both polysaccharides are involved in the avoidance of host innate immune mechanisms, such as resistance to phagocytosis, complement-mediated killing, and the bacterial activity of antimicrobial peptides, they are therefore essential for virulence. In addition, LPS is a major in the stimulation of adaptive immune response to infection.

Other explanation of immune response to *P. multocida* bacterin was recorded by **Gong et al. (2013)** who demonstrated that two Omp H and Omp A are the major immunogene antigens of avian *P. multocida,* which play an important role in reducing immune response that, confer resistance against infections. **Boyle and Finlay (2003)** concluded that the Omps promote adherence to host cell surfaces and are therefore likely involved in *P. multocida* virulence. Also, **Zang et al. (2013)** reported that the capsule is the major virulence factor of *P. multocida* serotype A:3 strain.

Challenge test among chicken groups were made here to study the correlation between level of immunity induced by locally prepared *P. multocida* inactivated vaccine and protection from the virulent strains.

Our results revealed that the clinical signs of *P. multocida* in vaccinated-challenged chicken groups were mild depression, off food, diarrhea, septicaemia and congested mucous membrane of conjunctiva and buccal cavity. Severe signs were observed in non vaccinated-challenged controls groups, while no signs were appeared in the non challenged control group during the observation period. Those results were in agreement with **Levy et al. (2013)** who recorded signs of depression, dullness, anorexia, greenish diarrhea and labored breathing in *P. multocida* challenged non vaccinated chickens, while the vaccinated birds did not show clinical signs except dullness and depression. Also, **Mehmood et al. (2016)** found that the clinical signs of FC in commercial layer flocks were depression, anorexia with ruffled feathers, mucous discharge from mouth and nares and cyanotic comb and wattles.

The mortality rates in different groups that recorded here were 15% and 20% in vaccinated-challenged birds with *P. multocida* type A1 and A:3; respectively, whereas, in non vaccinated-challenged controls groups, they were 90% and 80% for *P. multocida* type A:1 and A:3; respectively. No mortalities were observed in the non-challenged control group during the observation period. These results were in a partial agreement with **Mariana and Hirts (2000) and Levy et al. (2013)**.

The post-mortem examination of this study revealed mild and severe lesions in *P. multocida* vaccinated-challenged and non vaccinated-challenged control groups, respectively. The lesions included septicaemia, congestion of internal organs, enlarged liver with sub-capsular hemorrhage, pericarditis and enlarged and congested spleen. These lesions were supported by the results obtained by **Ashraf (2000)** who reported that the lesions of double dosed vaccinated and challenged birds with *P. multocida* were congested heart and slight congestion of the liver and spleen, while the lesions of *P. multocida* in non vaccinated-challenged birds were congestion of the subcutaneous blood vessels, dark red muscles, enlarged and congested liver and spleen and pericarditis. In addition,

Herath et al. (2010) recorded the gross lesions induced by vaccinal strain A:1 of *P. multocida* in chickens at 14 days post-challenge as severe congestion, hemorrhages, pericardial and peritoneum exudations, enlargement of spleen and liver with presence of necrotic foci over liver.

The results showed that vaccinated chickens were resistant to challenge with *P. multocida* A:1 and A:3 strains, where the protection rates were 85% and 80% respectively, while they were 10% and 20% in the non vaccinated-controls, respectively. These results correlated with **Fatma (2004)** who reported that two doses of prepared bacterin induced good protection (80-90%) against challenge with *P. multocida* of homologous immunogenic type but low protection (10-30%) against heterologous challenge. She proved that the locally prepared polyvalent bacterins should be used in cases of FC outbreaks, and the capsular antigen plays a little role in immunization when compared with the somatic antigen. **Jabbri and Moazeni Jula (2005)** demonstrated that inactivated trivalent FC vaccine consists of serotype 1, 3 and 4 *P. multocida* provided 70-80% protection in chickens against challenge with homologous strains. Also, **Ahmed et al. (2010)** detected that inactivated FC vaccine gave 100% protection in chickens against challenge with virulent strains of *P. multocida* type A and D. **Abdel-Aziz et al. (2015)** stated that inactivated FC vaccine induced high protection rates in chickens against challenge with virulent serotypes A:5 and D:2 (95 and 90; respectively). Furthermore, **Ali and Sultana (2015**) concluded that the protection rate was 100% in chickens vaccinated twice with alum- precipitated FC vaccine.

In this study, *P.multocida* could be reisolated from liver, heart blood and spleen of the chickens with high rate (90-95%) in non vaccinated-challenged and low rate (15-25%) in vaccinated-challenged birds. These findings supported by **Mahmoud (1999)** who found that the incidence of isolation of *P. multocida* was higher from non-vaccinated flocks than those from vaccinated ones. Partial agreement was recorded by **Jabbri and Moazeni Jula (2005)** who found that *P.*

multocida could not be recovered from immunized and challenged survived chickens, while it could be isolated from all dead or sick birds.

In our work, The microscopic lesions of group 1 (birds vaccinated with locally prepared inactivated *P. multocida* vaccine and challenged with *P. multocida* serotype A:1) revealed mild congestion of the central vein of liver. The heart showed normal myocardium and pericardium. Spleen showed mild congestion of the red pulp as well as depletion of the lymphoid follicle. In group 2 (birds vaccinated with locally prepared inactivated *P. multocida* vaccine and challenged with *P. multocida* serotype A:3), the histopathological lesions were hydropic degeneration of hepatocytes, severe congestion of central vein and moderate congestion of portal vein. Heart showed slight congestion of the myocardium, normal pericardium and normal endocardium. Spleen revealed slight congestion of the red pulp and slight hyper activity of the lymphoid follicle. The previous results were in partial agreement with **Ashraf (2000)** who noticed congestion with presence of some degenerative changes of liver in chickens which were vaccinated against FC and challenged with *P. multocida*. Spleen showed mild depletion of the lymphoid cells. Heart showed slight myocarditis in some chickens.

Histopathological lesions of groups 3 and 4 (non vaccinated and challenged with *P. multocida* A:1 or A:3 strain, respectively) were congestion of the portal vein of liver and the hepatocytes showed hydropic degeneration of cytoplasm with appearance of focal area of coagulative necrosis infiltrated with heterophils. The heart showed myocarditis with inflammatory cells infiltration and precardial and sub endocardial hemorrhages. Spleen showed congestion of the red pulp, focal area of necrosis and area of hemorrhage. These results agree with **Shilpa and Verma (2006)** who concluded that histopathological changes of local isolates of *P. multocida* A:1 in chickens were congestion, hemorrhages and mild degeneration of liver associated with necrotic changes involving groups of

hepatic parenchymatous cells with prominent heterophilic infiltration. Congestion and hemorrhages in heart, where the pericardium and myocardium were thickened with leukocytic infiltration and fibrinous exudates were also recorded. Spleen revealed congestion, hemorrhages and depletion of lymphoid elements. **Panna et al. (2015)** reported similar microscopic lesions of experimentally infected chickens with *P. multocida* that were include congestion with hemorrhages in heart, liver and spleen. Inflammatory cells in the pericardium of heart, and lymphocytic infiltration in central vein in liver were seen. **Hablovarid et al. (2009)** described the histopathological lesions of vaccinal strain of *P. multocida* A:1 in chickens as congestion and hemorrhages of the internal organs.

These lesions could be attributed to the direct effect of the endotoxin and ischemia which resulted from the bacterial emboli.

The antibiogram test for *P. multocida* serotype A:1 isolated strain was done *in-vitro* and the results showed sensitivity to chloramphenicol, tetracycline, trimethoprim /sulphamethoxazole, penicillin G, ofloxacin, norfloxacin, azithromycin, and erythromycin, while *P. multocida* serotype A:3 local strain was sensitive to cefoperazone, gentamycin, tetracycline, trimethoprim/ sulphamethoxazole, ofloxacin, penicillin G, chloramphenicole, norfloxacin, azithromycin, and erythromycin. Resistance to ampicillin and clindamycin was observed for both *P. multocida* serotypes A:1 and A:3.

In Egypt, similar results was detected by **Shimaa (2016)** who carried out antibiotic sensitivity test for 14 *P. multocida* isolates against 18 antimicrobial agents using disc diffusion technique and the result indicated that A1 isolates were sensitive to thiampheicol, sulfamethoxazole + trimethoprime, oxytetracyclin, streptomycin and neomycin. Also, **Ayman (2017)** reported that antibiotic sensitivity tests of *P. multocida* isolates serotype A:1 and A:3 were most sensitive

to norfloxacin (85.72%), ciprofloxacin (71.44%) gentamycin (66%), doxycycline (62%) and the highest level of resistance was detected to sulfadiazine 100%.

Sarangi and Panda (2011) studied the antibiotic sensitivity test of *P. multocida* isolates and found that the organisms were sensitive to enrofloxacin, gentamycin, levofloxacin, gatifloxacin, chloramophenicol and resistant to penicillin G, streptomycin, sulfadiazine, cephalexin, cephotaxim and ampicillin. Similar sensitivity were recorded by **Hirsh et al. (1989) and Shivachandra et al. (2004)** who found susceptibility of *P. multocida* to chloramphenicols, enrofloxacin, gentamycin, tetracycline, penicillin G, streptomycin and sulphonamide and trimethoprim. Moreover, **Kamruzzaman et al. (2016)** recorded that *P. multocida* isolates were sensitive to ciprofloxacin and azithromycin and intermediatly sensitive to gentamycin, tetracycline, amoxicillin and erythromycin.

Opposite result was obtained by **Victor et al. (2016)** who found resistance of *P. multocida*to ofloxacin, ciprofloxacin, enrofloxacin, furasol, ceftazidime and cefuroxime.

Atere et al. (2015) demonstrated that the multidrug resistance of *P. multocida* is attributed to multi-use of antibiotics as additives in feed and extensive use of antimicrobial agents by poultry flocks. Antimicrobial resistance of *P. multocida* has been linked to small plasmids **(Rosenau et al., 1991 and Rigobelo et al., 2013)** and the coexistence and spread of these small plasmids have resulted in multi-resistance of *P. multocida* isolates **(San Millan et al., 2009).**

Strains of *P. multocida* vary in their susceptibility to different chemotherapeutics. Variation in the sensitivity patterns among different studies may be due to over or limited previous exposure and/or indiscriminate use of antibiotics for prevention and control of the disease **(Kamruzzaman et al., 2016)**.

In this work, the antimicrobial resistance was law which might be due to that the isolated *P. multocida* doesn't acquiring resistance or undergoing selection pressure.

From the above mentioned results, it could be concluded that *P. multocida* strains are circulating in Egyptian layers and breeders chicken flocks inspite of vaccination and medication. So, continuous surveillance studies are important to detect the presence of the new strains of *P. multocida* in the field.

Moreover, preparation of inactivated vaccine from local *P. multocida* isolated strains is very important for prevention of the disease that causes severe losses in layers and breeders chicken flocks.

Continuous detection of sensitivity of *P. multocida* strains to different antimicrobials that used in the field is the must to avoid the problem of drug resistance during controlling of the disease.