Immuno-Gold Labelling of *Mycoplasma iowae* For Light Microscopy

Jalal M. Shareef
Dept. of Micro., Coll. Of Vet. Med., Univ. of Sulaimany,
Kurdistan – Iraq

SUMMARY

Mycoplasma iowae is an egg transmitted organism with serotypes previously known as I,J,K,N,Q and R. Some members of this group are embryo lethal for turkey poults and chicks and cause low hatchability. Culture methods have been known to be unreliable for monitoring M. iowae in natural infection due to intermittent spread and the unpredictable nature of this organism.

In the present work M. iowae was isolated from vaginal and cloacal swabs of a naturally infected turkey hen.

An immuno-gold light microscopy technique was utilized for identification of the organism in situ. The technique is being used to study the relationship between M. iowae and the tissues of the reproductive tracts of turkey.

Photographs of the histology of infected turkey hen reproductive system are presented, as well as photographs of gold-labelled M. iowae microcolonies close to epithelial cells in tissues.

INTRODUCTION

Mycoplasmas belonging to avian serovars I,J,K,N,Q and R are serologically related and are considered to belong to one group, which given species status with the name *Mycoplasma iowae* (1).

M. iowase is considered as an important poultry pathogen, it is embryo lethal for turkey and chicken (2,3). It has been isolated from the respiratory tract (4), the hock joint(5,3), the oviducts (6), and semen (7). It has been also isolated from the reproductive system of adult males and females (8), the cloaca of poults and mature turkeys (9,4), also from gastrointestinal tract of experimentally infected poults (10).

M. iowae may be spread venerelly (8). Poults with severe disease may show airsaculitis, skeletal abnormalities and retarded growth (11). In experimentally infected poults Shareef (12) reported stunting, poor feathering, chondrodystrophy, deviated toes, crooked neck and exposed elbow joints. More or less these symptoms were also reported by Bradbury (13). Pathological changes in the bursa of Fabricius was also observed by Shareef (12).

M. iowae is relatively resistant to many antibiotics (8), and a little information is available regarding the precise sites of *M. iowae* infection. The intermittent spread and unpredictable nature of *M. iowae* may obscured the monitoring methods particularly the culture methods (14). The localisation of *M. iowae* in host tissue by immuno-gold

technique would help understanding its pathogenesis.

Since the technique of pre-embedding immuno-gold labelling in transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) was developed and described previously (15,16,17) proved successful with experimentally and naturally infected tissues, it was used in the present study to examine tissue taken from naturally infected turkeys under the light microscopy.

Immuno-gold techniques have been used widely for localisation of antigens in light and electron microscopy (18,19). A number of different technique have been developed where the antibody has a marker attached to it so that the antigen-antibody complex can be visualised (Fig: 1 & 2).

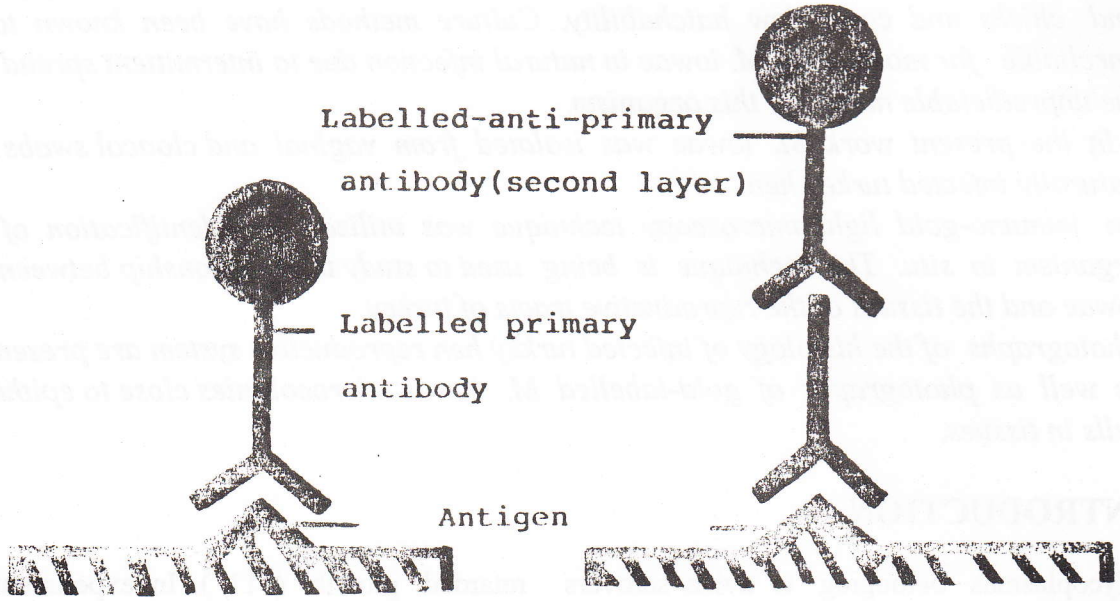

Fig. 1 Direct Immunolabel technique

Fig. 2 Indirect Immunolabel technique

These technique, including immuno fluorescent, immuno-gold ...etc., are used either as a direct (Fig:1) or indirect method (Fig:2) in which the primary antibody is unlabelled and to which is then attached a labelled secondary antibody. This technique is preferable since the immunoreactivity of the primary antibody can be preserved.

Immuno-gold or immunoglobulin adsorbed to colloidal gold, has found more widespread use recently in immuno-cytochemical techniques (20). One major advantage of this technique over traditional enzyme methods is that colloidal gold binds to macromolecules by non-covalent electrostatic adsorption, without changing the biological characteristic of the conjugated macromolecule. It is worthy also to mention that gold solutions can be prepared easily in a range of particle sizes (5-150 nm diameter) of reproducible consistency. Moreover gold probes are stable for relatively long period, they are flexible and predictable.

Under light microscopy, gold gives an identical signal, so the reactivity of the antibody or tissue can be checked under light microscopy even before electron microscope is attempted.

MATERIALS AND METHODS

a) Collection of tissue specimens

A white turkey hen at the end of the laying period was obtained from a flock known to be infected with *M. iowae*.
Swabs were taken from cloaca and vagina and plated directly on to mycoplasma agar and used to inoculate mycoplasma broth for incubation at 37°C.
The turkey was sacrificed, dissected, and portions of cloaca and vagina were separately placed in sterile petri dishes, and covered with tissue culture medium at 20°C. The medium was changed twice and the portions were cut into 5X5 mm pieces, then covered with fresh medium. Tissue culture medium was used to ensure preservation of tissue between collection and processing for light microscopy.
Antisera were raised in rabbits, using the type and field strains separately. Since the serotype of the expected mycoplasma was unknown, 2 types of purified anti *M. iowae* IgG { (type strain, R 2 Ig G PA) and (field strain S1 IgG PA) } were used. Purified anti *M. iowae* IgG were prepared by chromatography on PA-Sepharose CL-4B. Specified anti *M. iowae* IgG was omitted, as a control.

b) Pre-embedding immuno-gold labelling of *M. iowae*

The above tissue pieces were treated with equal volumes of 0.5 % glutaraldehyde in PBS, containing 0.002 M sodium phosphate, 0.145 M NaCl, pH 7.2 at 4°C for 15 min. Specimens were washed once in PBS containing 0.1 M NHCl and 3 times in PBS alone. They were nailed epithelial surface up to a slice of silicon rubber stopper with glass nails. Immuno-gold labelling was performed as follows:
The specimens were covered with 50 µl preimmune rabbit serum (diluted 1 in 20 Tris buffer) at 4°C for 30 min. The serum was drained. Purified anti M. iowae IgG { (R2 IgG PA, anti type strain) or (R1 IgG PA, anti field strain S1), diluted 1 in 10 in Tris buffer was added at 4°C for 30 min. Preimmune rabbit serum diluted 1 in 20 was used for controls. The specimens were washed 3 times in PBS, and covered with normal goat serum (diluted 1 in 20) at 4°C for 30 min. The serum was drained. 50 µl gold probe (Janssen GAR 40, Goat anti-rabbit IgG , diluted 1 in 20 in Tris buffer) was added at 4°C for 30 min. The specimens were washed 3 times PBS then fixed in 10% formal saline for light microscopy. The preparations were then processed and embedded in wax. Sections were cut at 5 µm and stained with haematoxylin and eosin.

RESULTS

positively as *M. iowae* by indirect immunofluorescence.

In the light microscopy both labelled and unlabelled mycoplasma microcolonies were seen in sections taken from cloaca and vagina, which had been treated with *M. iowae* specific antibody (plates 1 & 2).

Growth of mycoplasma colonies was observed after 72 hours incubation of the mycoplasma agar cultures which had been obtained from vaginal swabs, but not from the cloaca. The isolate was subcultured 3 times in mycoplasma broth, containing no bacterial inhibitors. Finally the isolate was cultured on mycoplasma agar and identified

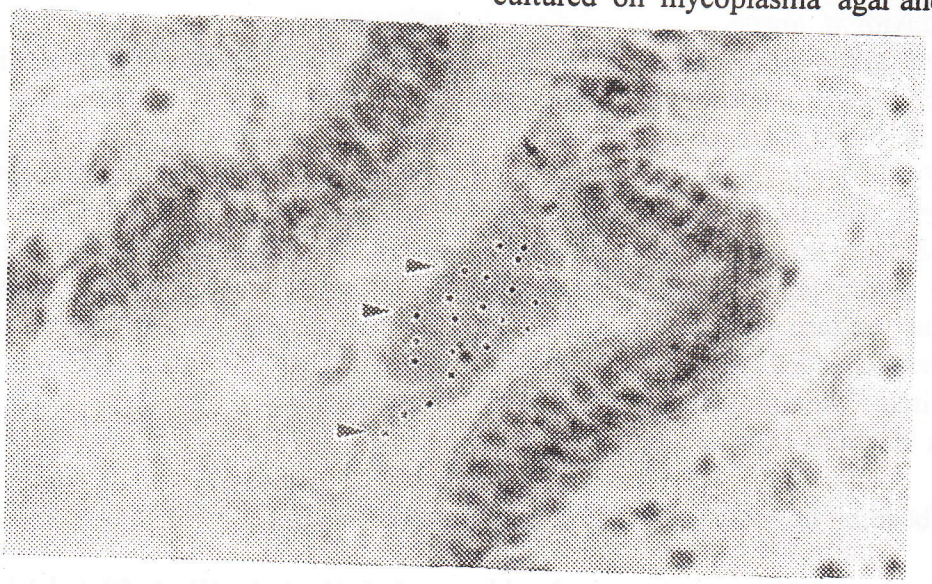

Plate 1. Section through vagina of turkey hen naturally infected with *M. iowae*, treated with anti-M.iowae IgG and gold probe, showing labelled mycoplasma microcolonies, aggregates of gold practicles (darts).

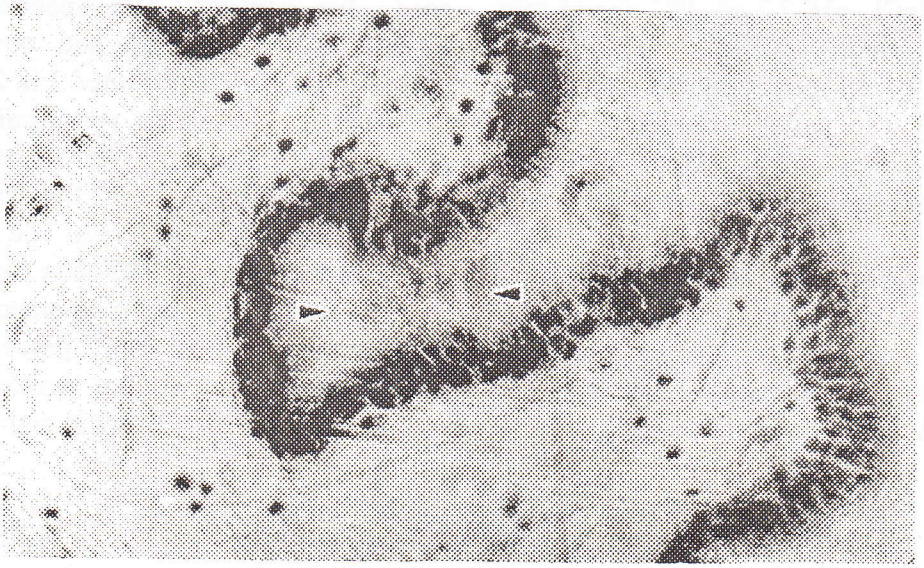

Plate 2. Section through vagina of turkey hen naturally infected with *M.iowae*, treated with preimmune serum and gold probe, showing unlabelled mycoplasma microcolonies (darts).

Microcolonies close to the epithelial cells, but not attached were observed in both cloaca and vagina, i.e no apparent contact was noticed between the microcolonies and the epithelial surface. In sections from vagina, labelled mycoplasma microcolonies with aggregates of gold practies were observed between the folds of vaginal epithelium, close to the tips of cilia. No labelled mycoplasma microcolonies were observed in sections taken from controls treated with preimmune serum only. Some deep cellular structures were not well preserved because of prolonged immuno-gold labelling prior to the fixation in formal saline.

DISCUSSION

This study has demonstrated the efficacy of pre-embedding immuno-gold labelling of *M. iowae* in the light microscopy. The staining results were consistent and reliable. The results which have been presented demonstrate that pre-embedding labelling of *M. iowae* cells in tissue is possible using the antiserum raised.

Gold particles were not observed in control preparations. Non-specific adsorption of the gold markers was very low because of precautionary measures described earlier. The homogenous dilution of gold probe is particularly important with this technique to reveal the distribution of the antibody binding sites.

The results of staining with gold probe indicate that the gold particles remained homogeneously distributed in the solution. This was probably because care had been taken with the pH, temperature, type of diluent buffer, and the dilution of gold probe (21). The degree of labelling is affected by several factors which include immunocytochemical staining procedure, particularly the size of gold probe and the pH of the washing water, as well as fixation, dehydration and the infiltration of wax.

Light glutaraldehyde fixation of bacterial specimens was recommended prior to immuno-gold labelling (22) in order to prevent antigen diffusion and/or stabilise antibody binding. Pre-fixed mycoplasma cells had previously been found to be better preserved and their antigens better stabilised (23). However in a study using ferritin-conjugated concanavalin A, Schiefer et al., (24) reported only weakly-labelled mycoplasma cells after treatment with glutaraldehyde. These authors explained that the result might be due to cross-linking or modification of mycoplasma surface proteins by glutaraldehyde. Positive immunolabelling was obtained from preharvesting fixation of *M. iowae* cells in 0.25% glutaraldehyde (17). The glutaraldehyde fixed mycoplasma cells were washed in PBS containing 0.1 M NHCl to block any unreacted aldehyde groups of the membrane-bound glutaraldehyde which might interfere with cell labelling (25). This was followed by washing in PBS and labelling with immuno-gold. Tap water of low pH is not recommended for washing, since it may affect the degree of immunocytochemical labelling by eluting bound antibody (26). PBS for washing was adjusted to neutral pH and filtered to preserve the quantity of conjugated antibody and to avoid contamination of the specimens. All reactions were carried out at 20°C, since non-specific reactions had been observed in specimens incubated at 4°C for 24 hours. Non specific background adsorption of gold probe was further reduced by using a Tris buffer containing gelatin, ovalbumin and Tween 20 (18).

This technique provides a permanent record of labelling, unlike the transient of immunofluorescence. Thus with a suitable antibody it would be particularly useful for the identification of mycoplasmas. A great advantage is that specimens which have been labelled pre-embedding can be examined by a light microscope for localisation of antigens.

Pre-embedding labelling is particularly suitable for detecting external antigens. It is concluded that this technique can be used for detecting external antigens on *M. iowae*. As long as mycoplasmas are growing outside host cells they can be considered to be extracellular antigens and they may be labelled by this technique. However if they are intracellular, pre-embedding will not be suitable and post-embedding labelling must be used. Post-embedding labelling can visualise target antigens either on the cell surface or within cells or tissues. Another advantage of the present technique is that, the antigens are not exposed to antibodies after they have been affected by fixation, dehydration and wax infiltration.

In spite of the satisfactory results obtained, several problems have been identified in the application of immuno-gold techniques.

The procedure should be carried out before loss of external cellular antigen, which often occurs with mycoplasmas due to age and pH.

To circumvent these problems, glutaraldehyde can be used for pre-fixation, but attention has to be paid to its concentration since antigenicity might be greatly reduced by high concentrations. This study has demonstrated that lowering the concentration of glutaraldehyde to 0.5 % for pre-fixation retains a high proportion of *M. iowae* antigens. After the immuno-gold reaction had taken place, a secondary fixation was carried out with a higher concentration of formal saline (10%), since a low concentration of the pre-fixation glutaraldehyde by itself is not sufficient to ensure good preservation of cell ultrastructure. One drawback of this technique might be loss or detachment of probe from the labelled specimen during the prolonged processing, since labelled cells have to be washed, dehydrated in ethanol and infiltrated with wax. Surviving internal antigens cannot bind to antibodies in the present technique.

In the present study *M. iowae* microcolonies were not found close to the epithelial cells of both cloaca and vagina, since this mycoplasma possess many long fibrils which are attachment organelles to the host epithelial cells (27).

The extension of pre-embedding immuno-gold labelling to involve more experimentally and naturally infected tissue may provide more significant results.

The applications and development of the colloidal gold marking system in microbiology will certainly continue to contribute to the growth of immunocytochemistry and to resultant discoveries.

It is of interest to determine whether *M. iowae* itself can be found within host cells, so this technique can be applied for studing the pathogenesis of *M. iowae* and answering more questions concerning this type of mycoplasma. As far as I am concerned the present work can be considered as the first successful technique, regarding the *M. iowae* pre-embedding immuno-gold labelling in the light microscopy within naturally infected tissue.

REFERNCES

1. Jordan, F. T. W., Erno, H., Cottew, G. S., Hinz, K.H., and Stipkovits, L. 1982, Characterisation and taxonomic description of five mycoplasma serovars (serotypes) of avian origin and their elevation to species rank and further evaluation of the taxonomic status of mycoplasma synoviae. INT J SYST BACTERIOL, 32, 108-115.

2. Yoder, H. W., Jr. and Hofstad, M. S. 1962, A previously unreported serotype of avian mycoplasma. AVIAN DIS, 6, 147-160.

3. Yoder, H. W., Jr. and Hofstad, M. S. 1964, characterisation of avian mycoplasma. AVIAN DIS, 8, 481-512.

4. Jordan, F. T. W. and Amin, M. M. 1980, A survey of mycoplasma infections in domestic poultry. RES VET SCI, 28, 96-100.

5. Bradbury, J. M. , McCathy, J. D., and Ideris, A. 1983, Bone, joint and tendon abnormalities in poultry following infection with *Mycoplasma iowae*. YALE J BIOL MED, 56, 852.

6. Rathore, B. S., Mohanty, G. C. , and Rajya, B. S. 1979, Isolation of mycoplasma from oviducts of chickens and their pathogenicity. INDIAN J MICROBIOL, 19, 19.

7. Shah-Majid, M. and Rosendal, S. 1986 a, *Mycoplasma iowae* from turkey phallus and semen. VET REC, 12, 435.

8.Jordan, F. T. W. 1985, Gordon memorial lecture: people, poultry and pathogenic mycoplasmas. BR POULT SCI, 26,, 1-15.

9.Jordan, F. T. W. 1983, Recovery and identification of avian mycoplasmas. In" Methods in Mycoplasmology" (Tully, J. G. and Rzin, S. eds.), II, Academic press, London. 69-79.

10.Shan-Majid, M. and Rosendal, S. 1986 b, The viability of Mycoplasma iowae at 40°C in turkey semen. POULT SCI, 65, 1631-1632.

11.Bradbury, J. M. and Ideris, A. 1982, Abnormalities in turkey poults following infection with *Mycoplasma iowae*. VET REC, 110, 559-560.

12.Shareef, J. M. 1983, Investigation into the effect of M.iowae on the immune system of the turkey. Report of a research project in partial fulfilment for the Degree of M.V.Sc. in Avian Medicine, University of Liverpool.

13.Bradbury, J. M. 1984, Effect of *Mycoplasma iowae* infection on the immune system of the young turkey. Isr. J. Med. Sci., 20, 985-988.

14.Grant, M. 1987, Significance, epidemiology and control methods of Mycoplasma iowae in turkeys. Ph. D thesis, Manchester Metropolitan University.

15. Shareef, J., Willcox, J., Kumar, P. 1986, Immunogold labelling of Mycoplasma iowae for electron microscopy. A poster was presented at "Microbe 86", XIV International Congress of Microbiology, September 1986, Manchester, U. K. Abstract P. 208, Poster No. 16-12.

16.Shareef, J. , Willcox, J., Kumar, P. 1988, Immuno-gold electron microscopy for identification of Mycoplasma iowae in experimentally infected turkey infundibulum. A poster was presented at 7th International Congress of the Internationa Organisation for Mycoplasmology, June 1988, Baden near Vienna, Austria. Abstracts, poster No. 158.

17.Shareef J. M. 1988, The development and application of immunogold labelling technique of Mycoplasma iowae in turkeys, Vol I, Vol II. Ph.D Thesis, Manchester Metroplitan University.

18.Beesley, J. E. 1985, Colloidal gold. A new revolution in marking cytochemistry. PROC R MICROS SOC J, 20, 187-196.

19.Roth, J. 1984, Light and electron-microscope localisation of antigens with the protein A-gold (PAG) technique., In "Advances in Immunohistochemistry" (Delellis, R. A.),Masson Publishing U.S.A, 43-65.

20.Romano, E. L. and Romano, M. 1984, Historical aspects, In: Immunolabelling for electron microscopy, eds polak, J. M. and Varendell, I.M. Elsevier Publishers BV, Amsterdam, 3-2

21.Habere, K. and Frosch, D., 1982, Lateral mobility of membrane-bound antibodies on the surface of Acholeplasma Laidlawii: evidence for virus induced cell fusion in a procaryote. J BACTERIOL, 1952, 471-478.

22.Beesley, J. E., Orpin, A. and Adlam, C., 1982, A comparison of immunoferritin, immunoenzyme and gold-labelled protein A methods for the localisation of capsular antigen on frozen thin sections of the bacterium, Pasteurella haemolytica. HISTOCHEMISTRY, 14, 803-810.

23.Schiefer, H-G, Krauss, H., Brunner, H., and Gerhardt, U. 1976, Ultrastructural visualization of anionic sites on mycoplasma membrances by polycationic ferritin. J BACTERIOL, 127, 461-468.

24.Schiefer, H-G, Krauss, H. , Schummer, U., Brunner , H., and Gerhardt, U. 1978 a, Studies with Ferritin-Conjugated concanavalin A on carbohydrate structures of mycoplasma membrances. FEMDS MICROBIOL LETT, 3, 183-185.

25.Kahane, I. And Schiefer, H-G, 1983, Characterisation of carbohydrate components of mycoplasma membrane In "Methods in Mycoplasmology" (Razin, S. and Tully, J. G. eds.), I I , Academic press, London, 285-299.

26.Beesley, J. E. , Orpin, A., and Adlam, C. 1984, An evaluation of the conditions necessary for optimal protein A-gold labelling of capsular antigen in ultrathin methacrylate sections of the bacterium Pasteurella haemolytica. HISTOCHEMISTRY, 16, 151-161.

27.Shareef, J., Willcox, J., Kumar, P. 1988, Adherence mechanism of *Mycoplasma iowae* to epithelial mucosa of the turkey cloaca. A poster was presented at 7th International congress of the International Organisation for Mycoplasmology, June 1988, Baden near vienna, Austria. Abstracts, Poster No. 66.

نیشانەکردنی *Mycoyplsma iowae* بە دژەتەنی ئالتونی لە ڕۆشنەووردبینیدا

جلال مجید شریف
کۆلیجی پزیشکی ڤیتێرنەری-زانکۆی سلیمانی

کورتە

Mycoyplsma iowae کە جاران ناوتەبرا بە پێی جۆرەکانی وەک I,J,K,N,Q,R یەکێکە لەو میکرۆبانەی کە تەگوێزرێتەوە بۆ جوجک لەناو هێلکەدا. هەندێک جۆری ئەم کۆمەلە هەرلەناو هێلکەدا جوجکی قەل و مریشک لەناوتەبەن و پێژەی هەلهاتنی هێلکەکەم دەکەنەوە. بەهۆی ئەوەی کە *M.iowae* بەشێوەی پچڕپچڕ و چاوەڕوان نەکراو بلاوئەبێتەوە، ڕێگای چاندن زۆر سودی نیە بۆ تاقیکردنەوە و چاودێری کردنی شێوەی بلاوبوونەوەی سروشتی.
لەم توێژینەوەیەدا توانرا بەهۆی چاندنەوە *M.iowae* بەدەست بهێنرێت لە هێلکە جۆگەی قەلێکی من کە بەسروشتی تووش بوبو. سەرکەوتوانە ڕەنگی دژەتەنی ئالتونی لەگەل ڕۆشنەووردبین بەکارهێنرا بۆ دەستنیشانکردنی *M.iowae* لە جێگای خۆیدا.
بەهۆی ئەم شێوە ڕەنگکردنەوە توانرا پەیوەندی نێوان *M.iowae* و شانەکانی کۆئەندامی زاوزێی قەل تاشکرا بکرێت.
لەئەنجامدا وێنەی فۆتۆگرافی شانە تووشبووەکانی کۆئەندامی زاوزێ لەگەل وێنەی کۆمەلە *M.iowae* کە نیشانەکراون بە ڕەنگی دژەتەنی ئالتونی، هەرلە جێگای خۆیاندا و نزیک بە خانەکانی ڕووکەشە شانەی زێ و کۆجن خراونەتەبەرچاو.

تاشير *Mycoyplsma iowae* في المجهر الضوئي بواسطة الصبغة المناعية المذهبة

جلال مجيد شريف
كلية الطب البيطري -جامعة السليمانية

الخلاصة

تعتبر *Mycoyplsma iowae* احدى الجراثيم التي تنتقل عن طريق البيض وكانت تعرف سابقا بعتراتها I,J,K,N,Q,R.تسبب بعض عثرات هذه المجموعة هلاكات في اجنة الديك الرومي والدجاج داخل البيضة وتؤدي الى انخفاض نسبة التفقيس.
تتميز *M.iowae* بانتشارها المتقطع والغير متوقع لذا فان طريقة العزل بواسطة اوساط زرعية غير مجدية لاختبار ومراقبة الانتشار الطبيعي للمرض.
تم عزل *M.iowae* من مسحات المهبل والمخرج لدجاجة رومية انثى مصابة بصورة طبيعية.
استعمل الصبغة المناعية الذهبة مع المجهر الضوئي في هذه الدراسة وذلك لتثبيت الجراثيم في مواقعها. لقد تبت نجاح هذه الطريقة لدراسة العلاقة بين *M.iowae* والانسجة الماخوذة من الجهاز التناسلي في الدجاجة الرومية.
تم عرض صور فوتوغرافية للانسجة المصابة من الجهاز التناسلي كذلك صور لمجاميع *M. iowae* مصبوغة بصبغة المناعية المذهبة وفي موقع الاصابة قريبة من خلايا الاغشية المخاطية.