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# Detection of Mycoplasma infection in cows with pneumonic lesions by conventional and PCR method and measuring the antigenic profile of the isolated mycoplasma

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## SUMMARY

Three different laboratory diagnostic tests were used for rapid and accurate diagnosis and typing of different Mycoplasma strains causing respiratory diseases in cows. A total of hundred lung samples from cows, with pneumonic lesions were collected from El-Basateen abattoir, and examined for the presence of Mycoplasma, by using isolation, polymerase chain reaction (PCR) and measuring the protein profile of the different isolates using (SDS- PAGE). The results indicted that Mycoplasma bovis showed the highest percentage among other mycoplasma isolates (*M. arginin* and *M.bovirhinis* and untyped strains). The result reported that the PCR was more rapid, sensitive and specific for accurate detection of Mycoplasma infection in comparison with culture method that was more specific, most difficult and laborious of time and effort. The presence of specific protein bands at 112, 96, 65, 50, 41, 30, and 17 KDa in M.bovis isolates was characteristic. Slight differences in the expression levels of some proteins could be detected.

## INTRODUCTION

Mycoplasma can cause a variety of clinical and pathological signs in cattle, *M.mycoids* subsp.mycoids SC is the causative agent of contagious bovine pleruropneumonia. or associated with bovine respiratory tract as normal flora or as pathogens (e.g., *M.bovirhinis, M.bovoculi, M.arginini* and *M.bovigenitalium*) while, *M.bovis* causes bovine pneumonia, mastitis, abscesses, infectious keratoconjuctivitis and genital tract diseases(Alberto et, al.2006).

Mihai, et, al.(2006) mentioned that,

*M.bovis* has the ability to persist and to develop chronic pneumonia in the face of an immune response and prolonged antibiotic therapy, becuse it has the ability to undergo antigenic variation by phenotypic alteration of the immunodominant surface lipoproteins and modulates the host immune response, also it can adhere to, invade and survive in the epithelial and inflammatory cells.

Membrane surface proteins play a fundamental role in the pathogenesis of mycoplasmas; and the attachment of mycoplasmas to host cells is mediated by cytoadhesins (Razin et al., 1998). The minute mycoplasmas possess an impressive capability of maintaining a surface architecture that is antigenically and functionally placed the mycoplasmas in the group of bacterial pathogens and parasites distinguished by remarkable antigenic variability (Rasmussen et al., 1992 ; Robertson and Meyer, 1992). The proteins of Mycoplasma cells of various species produce highly reproducible and species-specific electrophoretic patterns in polyacrylamide gels. These electrophoretic patterns can be used for the rapid identification and classification of Mycoplasma (Razin and Rottem, 1967). Advances in polymerase chain reaction (PCR) technology have greatly improved the detection of mycoplasmas (Nicholas, 2002). The aim of this study was to apply the rapid specific and sensitive methods to diagnose and type the Mycoplasma infection causing pneumonia in cows.

## MATERIAL AND METHODS

### Mycoplasma studies

**I-Samples:** A total of hundred lung samples from cows, with pneumonic lesions were collected from El-Basateen abattoir from September 2008 till August2009 and prepared according to Razin, (1978). The prepared samples were coded and stored at $-20^0$C till examination, for isolation and identification of Mycoplasma

**II-Mycoplasma isolation and identification:**

**1- Liquid and solid media** were used for the isolation and propagation of mycoplasma was prepared as described by Sabry and Ahmed (1975).

**2-Digitonin sensitivity test** was done for the obtained isolates according to Erno and Stipkovits (1973) to differentiate between Mycoplasma and Acholeplasma species.

**3-Biochemical characterization** was carried out by glucose fermentation and arginine deamination tests and film and spot formation test as described by Erno and Stipkovits (1973)..

**4-Serological identification** was conducted by growth inhibition test as described by Clyde (1964). Standard antisera - locally prepared- were kindly supplied by Chief Researcher Dr. Nabil Al-Zeftawi, Mycoplasma Department,

Animal Health Research Institute, Dokki, Giza Governorate, Egypt.

**III-- Detection of Mycoplasma using Polymerase chain reaction (PCR):**

**a) Preparation of samples for DNA extraction** (Yleana et al., 1995): 5ml of a 24 hour broth cultures of samples were centrifuged for 10 minutes at 12000 r.p.m. The pellet was washed twice in 1 ml of phosphate buffered saline pH 7.2 (PBS) and suspended in 50 µl PBS. The cell suspension was heated directly at 100°C for 10 min. in a heat block to break the cell membranes, and then cooled on ice for 5 min. Finally, the cell suspension was centrifuged for 5 min. and the supernatant containing chromosomal DNA was collected and stored at -20°C until used.

**b)Primers used for detection of *M.bovis*** (Yleana et al., 1995): Two oligonucleotide primers were selected for the detection of *M.bovis*. The sequence of primer (1) was: 5\-CCT TTT AGA TTG GGA TAG CGG ATG-3\. The sequence of primer (2) was: 5\- CCG TCA AGG TAG CGT CAT TTC CTA C-3\. the primer was prepared by Sigma.

**c) Procedure for DNA amplification** (Yleana et al., 1995): PCR amplification was performed in 50 µl reaction mixture consisting of 5 µl of 50 ng from isolated mycoplasma genomic DNA, 10 µl of 10 x Taq buffer (10mM tris- HCl [pH 8.8], 50 mM KCl), 1 µl of 50 pM of each primer, 1.5 mM MgCl2, 1 µl of 2U of Taq thermosTable DNA polymerase, 1 µl of 50 uM of each dNTP, and 31µl of DNase- RNase- free, deionized water. DNA amplification was carried out in PTC-100 programmable thermal controller (MJ, Research Inc.). The thermal profiles were as follows: Denaturation at 94 oC for 45 seconds, primer annealing at 60oC for 1 min., and extension at 72oC for 2 min.the amplifications were performed for 30 or 35 cycles with a final extention step at 72oC for 3 min. After the reaction, the amplified DNA was electrophoresed on 1.5% agarose gel for 90 min. at 100 volts, DNA Ladders: 100 bp (Pharmacia), Cat. No. 27-4001- 01, USA was added then stained with ethidium bromide. After electrophoresis, the gel was visualized by UV transillumination and photographed. Image analysis was made by ImageQuantTL-V2003.03 (Amersham Biosciences).

**d)Primers used for detection of *M.arginini*** (Van Kuppeveld et. al,1994) oligonucleotide primers were selected for the detection of *M.arginini*. The sequence of primer (1) was: 5\-GGAGCAAACAGGATTAGATACCCT-3\. The sequence of primer (2) was: 5\-GCACCATCTGTCACTCTGTTAACCTC-3\. the primer was prepared by Sigma.

**e) Procedure for DNA amplification according to** (Van Kuppeveld et. al,1994)

**III-Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE):** Cells were harvested from aliquots (100 ml) of late log phase of cultures by

centrifugation at 12000 RPM for 30 min at 4oC. Cell pellets were washed three times in 0.1 M Phosphate Buffer Saline (PBS) pH 7.2 before carrying out protein estimation by Bradford (1976). Samples were boiled for 5 min in sample buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol,2%β-mercaptoethanol,0.1%bromophenol blue) and proteins separated on 10% acrylamide gels by SDS-PAGE applying 3 V/ cm for 2 h using Hoefer Scientific Instrument. Gels were stained by 1%Coomassie brilliant blue R and destained with mixture of, 45% methanol and 10% acetic acid (Laemmli, 1970). SDS-PAGE Molecular Weight standards, Low range (cataloge number 161-0304, BIORAD) was used as marker. Image analysis was made by image QuantTL-V2003software (Amersham Bioscience).

## RESULTS

### Results of Mycoplasma diagnosis

Distinct Mycoplasma colonies were observed in culture seeded with pneumonic lungs, The recovery rate of Mycoplasma from pneumonic lungs of cows was 26%(26/100).Twenty four isolates 92.86% (24/26)were Mycoplasma (digitonin sensitive), two isolates 7.14%(2/26) were Acholeplasma (digitonin resistant). Mycoplasma were further typed biochemically and serologically as shown in table (2).

**Table (1):** Total recovery rate of Mycoplasma from the examined samples and digitonin sensitivity test of the obtained isolates

| SAMPLE | RECOVERY RATE | | | DIGITONIN SENSITIVITY | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Sensitive *MYCOPLASMA* | | Resistant *ACHOLEPLASMA* | |
| | Exam. | No.+ | %+ | Exam | No.+ | % | No.- | % |
| Lung tissues from Cows | 100 | 26 | 26% | 26 | 24 | 92.86% | 2 | 7.14% |

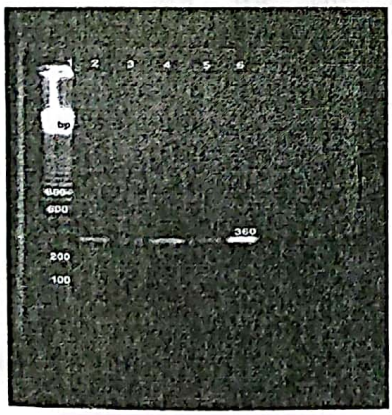
Table (2): Biochemical characterization and serological typing of the obtained Mycoplasma isolates

| SAMPLE | NO. EXAMINED | BIOCHEMICAL TYPING | | | SEROLOGICAL TYPING |
|---|---|---|---|---|---|
| | | G | A | F & S | |
| Lung tissue of cows | 24 | -ve | +ve | -ve | 6 *M. arginini* |
| | | +ve | -ve | -ve | 2 M.bovirhinis |
| | | -ve | -ve | +ve | 10 M.bovis and 6 Un typed strain |

G = glucose fermentation (the change of the color from red to yellow or orange is considered positive)
A= arginine hydrolysis (the change of color from red to pink is considered positive)
F&S= film and spot formation (the observation of pearly area clearing around area of growth is considered positive).

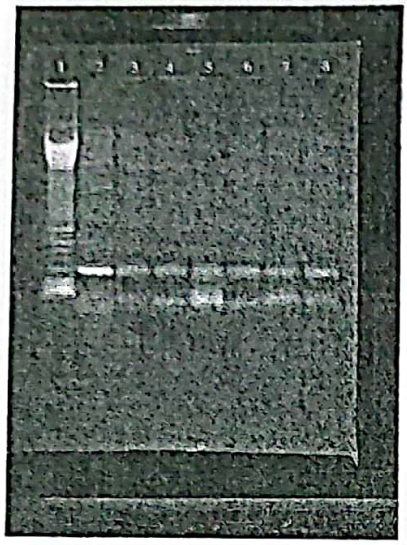
PCR analysis was performed on the lung samples using primers specific for M.bovis and primers specific for *M.arginini* with appropriate positive PCR control. Ten samples gave a specific band at 360 bp which is characteristic for *M.bovis*; all other samples gave negative results. Other six samples gave a specific band at 280 bp which is characteristic for *M.arginini* (Figs. 1 and 2).

Fig. (1): Electrophoretic analysis of PCR products of *M bovis* reference strain and the field isolates.




1-100 bp DNA ladder
2-5 Field isolate gives specific band at 360bp
6-*M.bovis* reference strain gives specific band at 360bp

Fig. (2): Electrophoretic analysis of PCR products of *M.arginini* reference strain and the field isolates.



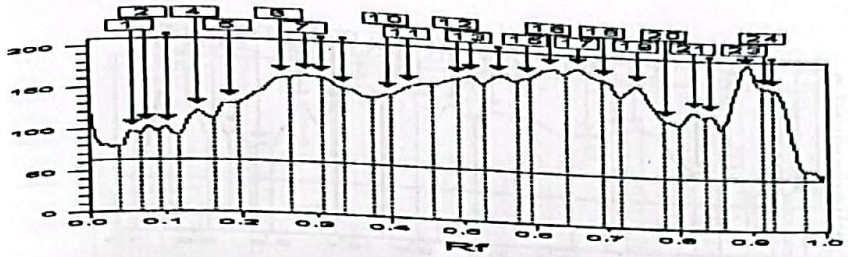
1-100 bp DNA ladder
2 -M.arginini reference strain gives specific band at 280 bp
3-8 Field isolate gives specific band at 280 bp

**Table(3):** The protein and antigen profiles of 10 isolates of *Mycoplasma bovis* were compared to the reference *M.bovis* strain by sodium dodecyl sulfate-polyacrylamide gel electrophoresis

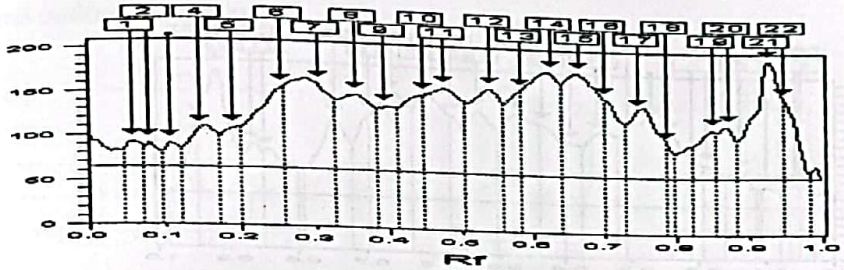
| | Lane 1 | Lane 2 | Lane 3 | Lane 4 | Lane 5 | Lane 6 | Lane 7 | Lane 8 | Lane 9 | Lane 10 | Lane 11 | Lane 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | MW (kd) | MW (kd) | MW (kd) | MW (kd) | MW (kd) | MW (kd) | MW (kd) | MW (kd) | MW (kd) | MW (kd) | MW (kd) | MW (kd) |
| 2 | 112.16 | 116.45 | 108.37 | 116.85 | 116.85 | 120.07 | 113.7 | 114.09 | 115.66 | 112.16 | 118.05 | 94 |
| 3 | 108.37 | 111.39 | 103.27 | 112.16 | 110.63 | 114.09 | 112.16 | 111.39 | 112.16 | 107.63 | 112.54 | 67 |
| 4 | 103.27 | 109.12 | 95.66 | 107.26 | 70.1 | 109.12 | 108.75 | 109.5 | 108.37 | 102.55 | 107.26 | 43 |
| 5 | 96.33 | 94.67 | 90.76 | 96 | 63.92 | 104.34 | 103.62 | 96 | 105.43 | 95.66 | 104.34 | 30 |
| 6 | 89.18 | 88.87 | 85.17 | 89.18 | 47.18 | 96 | 96.67 | 87.62 | 96.67 | 88.24 | 99.4 | 21.1 |
| 7 | 78.45 | 86.69 | 77.89 | 77.61 | 42.67 | 88.55 | 88.24 | 79.3 | 79.87 | 78.45 | 87.62 | 14.4 |
| 8 | 73.78 | 78.73 | 70.36 | 71.66 | 36.42 | 74.85 | 85.17 | 75.13 | 76.22 | 71.66 | 76.5 | |
| 9 | 70.88 | 70.1 | 65.36 | 66.34 | 33.12 | 71.66 | 80.16 | 65.6 | 67.32 | 65.12 | 71.66 | |
| 10 | 67.07 | 66.09 | 59.07 | 55.79 | 30.72 | 65.85 | 75.95 | 59.97 | 56.65 | 60.2 | 66.34 | |
| 11 | 59.74 | 62.5 | 55.16 | 53.07 | 29.1 | 54.31 | 70.88 | 54.73 | 51.25 | 54.31 | 62.27 | |
| 12 | 56.44 | 56.01 | 50.45 | 48.13 | 26.89 | 50.45 | 65.12 | 51.25 | 45.88 | 51.25 | 59.74 | |
| 13 | 49.47 | 50.26 | 46.99 | 43.54 | 22.25 | 44.97 | 60.42 | 44.97 | 40.78 | 44.79 | 57.74 | |
| 14 | 47.56 | 47.18 | 43.72 | 41.12 | 20.29 | 40.62 | 55.37 | 41.29 | 38.64 | 41.12 | 52.05 | |
| 15 | 44.07 | 42.84 | 38.48 | 36.58 | 18.43 | 37.36 | 51.25 | 38.16 | 35.35 | 37.52 | 48.32 | |
| 16 | 40.28 | 40.45 | 37.2 | 34.6 | 16.76 | 33.71 | 45.15 | 34.15 | 32.4 | 34.3 | 41.63 | |
| 17 | 37.68 | 37.2 | 34.15 | 30.86 | | 31.27 | 41.8 | 31.98 | 29.1 | 31.55 | 37.68 | |
| 18 | 34.6 | 34.3 | 32.69 | 27.79 | | 28.05 | 37.84 | 28.57 | 22.59 | 28.44 | 35.65 | |
| 19 | 31.98 | 32.26 | 31 | 26.14 | | 24.21 | 34.3 | 21.04 | 21.37 | 25.77 | 34 | |
| 20 | 28.7 | 31 | 27.92 | 22.36 | | 22.36 | 31.84 | 17.63 | 18.23 | 22.03 | 32.55 | |
| 21 | 26.14 | 28.05 | 26.89 | 21.15 | | 20.82 | 28.31 | 16.38 | 17.05 | 20.82 | 30.31 | |
| 22 | 23.62 | 22.7 | 24.8 | 18.73 | | 18.33 | 22.03 | | | 17.93 | 28.31 | |
| 23 | 22.36 | 19.04 | 22.82 | 17.44 | | 17.14 | 20.93 | | | 16.76 | 26.27 | |
| 24 | 19.66 | 17.93 | 21.15 | | | | 18.23 | | | | 24.21 | |
| 25 | 18.33 | | 18.93 | | | | 16.86 | | | | 22.03 | |
| 26 | 17.63 | | 17.63 | | | | | | | | 21.15 | |
| 27 | | | | | | | | | | | 18.03 | |
| 28 | | | | | | | | | | | 16.95 | |

Lane1 to 10 demonstrate the protein profile of the isolates.
Lane 11 demonstrates the protein profile of the reference *M.bovis* strain.
Lane 12 SDS-PAGE Molecular Weight standards, Low range

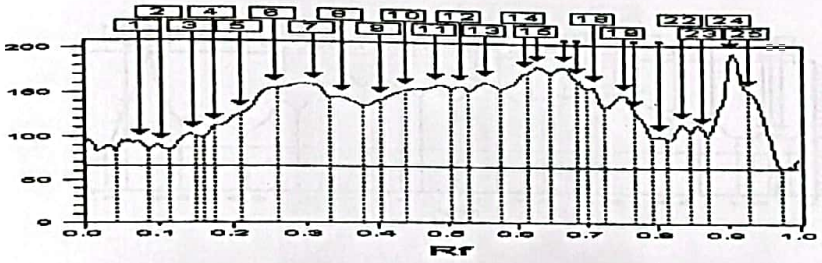
SDS PAGE analysis of *M. bovis* reference strain have reveled 28 protein bands ranged from 118.05-16.95 KDa, while, field isolates of M. bovis from cow gave 16- 26 bands ranged from 120.07 – 17.63 KDa .Cow field isolates of M.bovis mostly share with reference strain in the protein profile at 112, 96, 65, 50, 41, 30, and17 KDa , while isolate no. five showed some differences it had smaller number of bands only 16 bands and lake band at 50KDa.



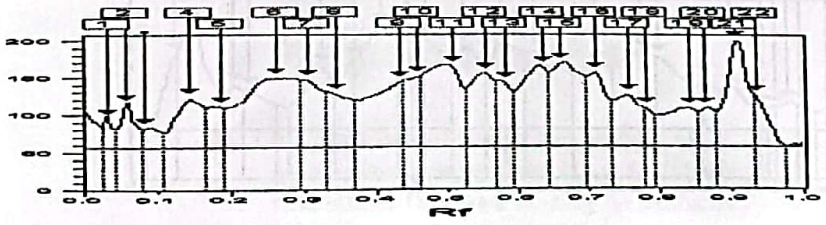

Lane 1



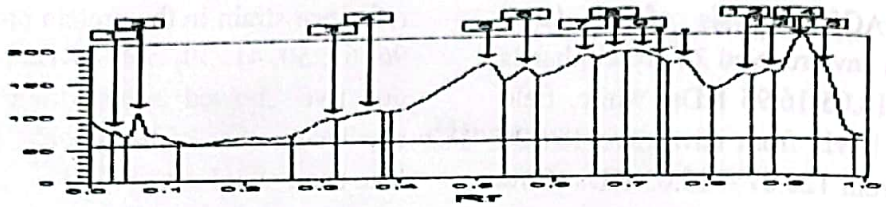

Lane 2



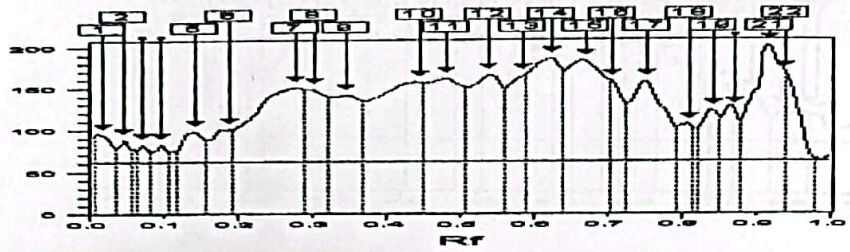

Lane3



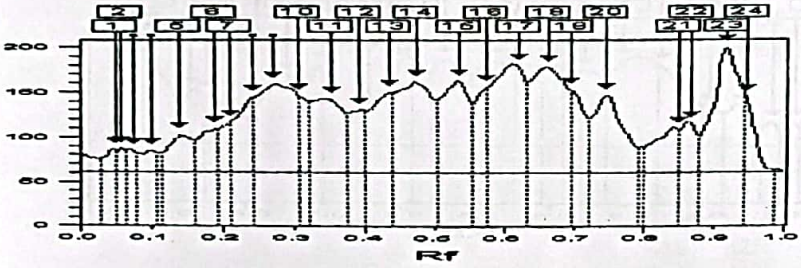

Lane 4



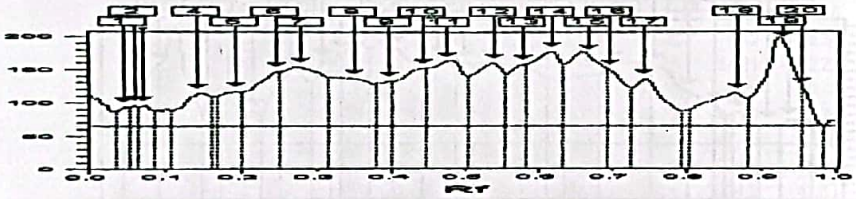
Lane 5



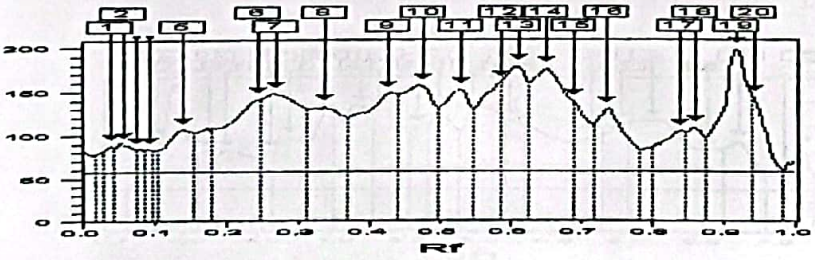
lane 6



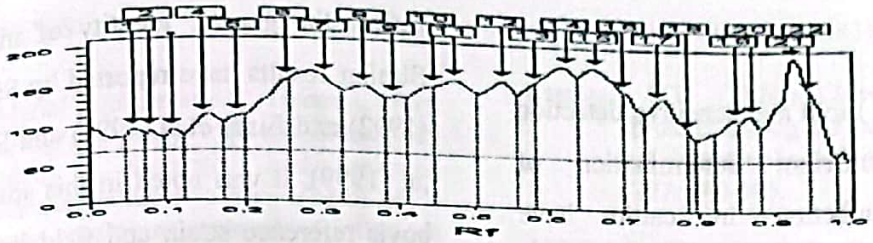
Lane7



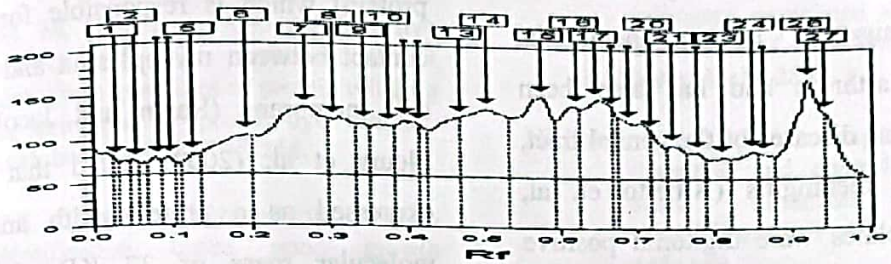
Lane 8



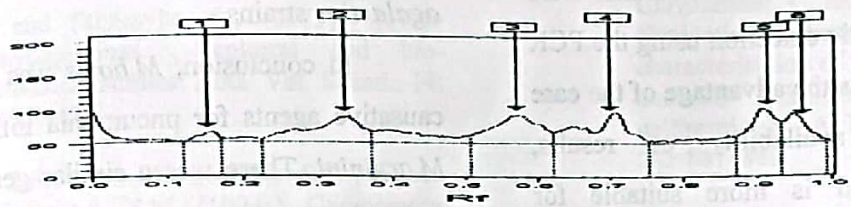
Lane 9



Lane10



Lane11 control positive M.bovis strain



Lane 12 The Molecular weight marker

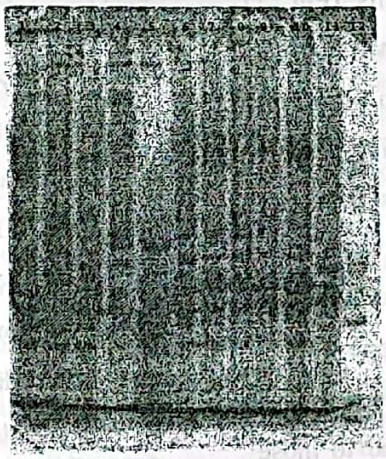


**Fig. (3)** Comparison of the protein analysis of *M. bovis* reference strain (lane 11) and *M. bovis* field isolates (lane1-10) showing the similarity in most bands using SDSPAGE

## DISCUSSION

The need for a rapid and sensitive detection method for efficient determination of Mycoplasma infection in cattle have developed several PCR assays that based on the 16S rRNA. *M.bovis* is a major and often overlooked cause of bovine pneumonia, mastitis and arthritis and has also been reported to cause diseases of the genital tract, abscesses and meningitis (Alberto et. al, 2006). Ten isolates were digitonin positive and proven to be *M. bovis* by biochemical and serological techniques. These results were confirmed by genetic detection using the PCR technique which has the advantage of the ease of use, rapid availability of results, standardization and is more suitable for processing large number of specimens (Foddai et al., 2005). Our results (Fig. 1) revealed the presence of *M.bovis* specific band at 360 bp which agreed with the results reported by Yleana et al., (1995). The genome detection of *M. arginini* using conventional PCR revealed a common band between field isolates of cow at 280bp and the reference strain. As described by Van Kuppeveld et, al.(1994). The electrophoresis profile of *M. bovis* field isolates is almost similar to those of reference strains with few exceptions that reflect the genetic identity of mycoplasma. Similar results have reported by Sachse et al. (1992) and Eissa et,al.(1994) and El Shater et ,al (1999). It was noted in this study that M. bovis reference strain and field isolates have the immunodominant protein P40 (adhesin protein) which is responsible for the close contact between mycoplasma and the target cell membrane (Razin and Jacobs, 1992). Fleury et al. (2002) stated that P40 was expressed as a protein with an apparent molecular mass of 37 KDa on sodium dodecyl sulfate-acrylamide gels by all *M. agalactiae* strains.

In conclusion, *M.bovis* was the main causative agents for pneumonia followed by *M.arginini*. There was a similar genetic and antigenic relatedness between *M.bovis* isolates as the genetic detection by PCR were expressed as proteins which appear by SDS-PAGE. *Mycoplasma bovis* strains from cattle came to slaughter at El-Basateen abattoir showed remarkable homogeneity with some exception that characterize each strain. Further studies involving more strains from different known localities are suggested to have more data on the genetic relatedness between strains and to facilitate epidemiological monitoring and control of the infections.

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# تحديد عدوى الميكوبلازما بالطريقة التقليدية وبأختبار تفاعل انزيم البلمرة المتسلسل فى أبقار تعانى من أعراض رئوية وقياس الصورة البروتينية للميكوبلازما المعزولة

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تم استخدام ثلاثة طرق تشخيصية مختلفة للتشخيص السريع الدقيق مع التصنيف للمعزولات التى تسبب أعراض تنفسية فى الابقار. في هذة الدراسة تم تجميع عدد 100 عينة من أابقار مصابة بأعراض تنفسية من مجزر البساتين لفحص وجود الميكوبلازما المسببة لهذه الأعراض. عن طريق عزل الميكروب و توضيح مدي إرتباط المعزولات من مصادر مختلفة عن طريق صورة البروتين وتفاعل أنزيم البلمرة العشوائي . وقد اوضحت الدراسة ان ميكوبلازما بوفيس اظهرت اعلى نسبة فى العزل بالمقارنة بالمعزولات الاخرى (ميكروب ميكوبلازما أرجينيني و ميكروب ميكوبلازما بوفيرينسس، كما تم عزل ميكروبات ميكوبلازما غير مصنفة ). وقد اثبتت الدراسة ان تفاعل أنزيم البلمرة العشوائي كان اكثر سرعة وحساسية وتخصص للتشخيص الدقيق للميكوبلازما بالمقارنة بطريقة العزل التى كانت اكثر تخصصا ولكن اكثرفى الصعوبة والمجهود والوقت.)وايضا تم توضيح مدي إرتباط المعزولات من مصادر مختلفة عن طريق صورة البروتين. وكانت وجود الحزم البروتينية عند 112-96- 65-50-41-30و17 كيلودالتون مميزاً للميكوبلازما بوفيس. وقد أظهرت العترات المفحوصة تجانساً ملحوظاً بين عترات ميكوبلازما بوفيس مع وجود بعض الاختلافات فى المستوى التعبيرى للبروتينات .