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USING MULTIPLEX PCR FOR DETECTION OF SOME MICROBIAL PATHOGEN CAUSING PNEUMONIA IN CAMEL

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ABSTRACT
Pneumonia is one of the most important causes of morbidity and mortality, which cause severe economic losses in camels (Camelus dromedarius). This study was formed to compare the fast-responsive PCR with conventional culture method in pneumonia and evaluate the reliability of multiplex PCR assay (mPCR) for simultaneously detecting and identifying 6 pathogenic bacteria. So, 160 nasopharyngeal swabs were collected from 160 camels from Matrouh and Alexandria Governorates, Egypt, suffering from pneumonia. The result revealed that (36.8%) and (51.2%) isolates were obtained by culture and PCR respectively. The isolates constituted 6 genera of pathogenic bacteria, were as follows, Klebsiella spp., (12.5%) and (16.2%), E. coli, (10.6%) and (13.1%), Staphylococcus spp., (9.4%) and (12.5%), Salmonella spp., (2.5%) and (4.3%) and Pasteurella multocida (1.9%) and (3.1%) by culture and PCR respectively to the total number of isolates. Meanwhile, Mycoplasma spp., (1.9%) were detected only by PCR. Multiplex PCR assay found to be rapid, economic and sensitive tool for accurate detection of the 6 pathogenic organisms concurrently. In addition, mPCR detected virulence genes of these pathogens enhancing evaluation of the pathogenicity of pathogenic bacteria present in infected camels and is a useful and rapid technique to apply to field samples.

KEYWORDS: *Camelus dromedaries*, pneumonia, Culture, mPCR.

1-INTRODUCTION
Nowadays, some concern and care have been focused on camel due to its unparalleled adaptive characteristics for survivability in the difficult and harsh environment.[1] especially in the arid and semiarid areas. Camel has been for a long time an ignoring species,[2] but recently, camel research achieved more attention worldwide. Globally, except for some parasitic diseases, there is only a few information on camel diseases.[3] Respiratory diseases are encountered as an emerging health hazard to camel population worldwide due to significant mortalities and the high cost of vaccination and treatment.[4] The main etiology of most respiratory diseases of camels has not yet been fully determined. Additionally, respiratory diseases have emerged among camel worldwide causing morbidity, mortality, and loss of production.[5-6]

A set of infectious bacterial pathogens caused pneumonia in camel, play an important role in economic losses in its industry which include. *Pasteurella multocida*, the main cause of hemorrhagic septicemia disease (Pasteurellosis) in camels[7,8] which was considered a dangerous disease has high effects on the productivity of camel and may even cause high mortality. Infected animals may die within a few days of the begin of clinical signs, and the rest animals who still a life may become chronically infected.[4,9]

Klebsiella spp. is often cause sepsis, urinary and respiratory tract infections, mastitis and meningitis.[10] Pulmonary infections due to *K. pneumoniae* are characterized by a rapidly progressive clinical course complicated by lung abscesses and cause high mortality and morbidity if still camel untreated.[11]

Mycoplasmas are considered from the smallest free-living microorganisms, from class Mollicutes and are highly fastidious bacteria, difficult to culture and slow growing. Its highly contagious organism, capable of auto- replication[12] and cannot be discover during the period of incubation, besides, the serological cross-reactions among the Mycoplasma spp. are a stringent problem.[13] Many species are also important veterinary pathogens causing many different diseases as pneumonia.[14] Other pathogens such as Salmonella spp, Escherichia coli, and Staphylococcus spp. have been incriminated to threaten camel health throughout the world.[15,16,17]

The classical procedure for detecting and identifying pathogenic organisms are taking too much time and

complicated by serological cross-reaction between related organisms. Another problem can be caused by bacterial contamination of samples, as this usually prevents the growth of some of these organisms, especially, some bacteria such as. *P. multocida* do not have an effective selective medium.[18]

In view of these difficulties, faster, simpler, and less hazardous and more specific and sensitive diagnostic methods are needed for identification of these organisms. Polymerase chain reaction (PCR) with specific primers can achieve rapid and specific diagnosis of organisms in field samples in both acute and chronic infections.[19,20] In addition, PCR is more specific, sensitive for detection of multiple organisms and give fast and accurate results, but a large number of individual PCR assay are necessary if single primer sets are used on a large number of clinical samples.[21] So, the detection of many pathogens with a multiplex PCR (mPCR) approach would be relatively rapid, cost-effective and has been widely applied to detect multiple bacteria in clinical specimens.[22,23] In Egypt, camel is the important domestic animal in the deserts because of its adaptation to different climatic conditions and to the Lack of water and forage. It gives meat, milk, means of transport and plays an important role in the socio-cultural structure of the community. On the other hand, the respiratory infections have received little attention in camel outbreaks in Matrouh and Alaxandria Governorate, and no study has reported on the simultaneous detection of the six bacterial pathogens in a single tube. Therefore, the first aim of the current study was to compare the fast-responsive PCR method with the conventional culture method in respiratory infection and to evaluate the reliability and effectiveness of the mPCR method. The second was to identify which bacterial pathogens are the most common causes of respiratory infection in camel at the two Governorates.

2- MATERIALS AND METHODS

2.1. Animals

One hundred and sixty camels aged from 3 to 7 years with clinical signs of pneumonia were subjected to examination in this study. The samples were collected from Matrouh and Alexandria Governorates Egypt, for detection and identification of bacterial microorganisms causing pneumonia.

2.2. Samples

A total of 160 nasopharyngeal swabs were taken under aseptic conditions on transport media and sent in sterile bags in an ice box for bacteriological examination.

2.3 Bacteriological Isolation

The nasopharyngeal swabs cultured in selenite F broth, tryptic soy broth (Merck, Darmstadt, Germany), brain heart infusion broth, LB broth and modified Hayflick's broth and then incubated at 37°C for 18-24 hours. A loopful from inoculated broth was streaked onto the surface of S.S. and XLD agar plates, nutrient agar, MacConkey agar ,brain heart infusion agar, blood agar base supplemented with 7% sheep blood, then incubated at 37°C for 24-48 hours and modified Hayflick's Agar incubated at 37ºC in humidified candle jar for two weeks and examined microscopically using stereomicroscope (10X,Olympus CKX41) every 2-3 days until fried egg colonies appeared, followed by purification of the isolates according to Rosendal[24] and Sabry.[25] The obtained bacterial isolates were stained with Gram stain and examined microscopically, then fully confirmed by full biochemical identification according to Quinn[26] and Holth.[27]

2.4. DNA extraction

DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

2.5. Oligonucleotide Primer

Primers used were supplied from Metabion (Germany) are listed in (table 1).

2.6. PCR amplification

2.6. a. For uniplex PCR, primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix **(**Takara, Japan), 1 µl of each primer of 20 pmol concentration, 5.5 µl of water, and 5 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler. The products of PCR were separated by electrophoresis on 1% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the products were loaded in each gel slot.

2.6.b. For multiplex PCR, primers were utilized in a 50-µl reaction containing 25 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 7 µl of water, and 6 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler. The products of PCR were separated by electrophoresis on 1% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 40 µl of the products were loaded in each gel slot.

Organisms

The Organisms which was used as Positive control kindly taken from desert research center. (*Staph. Spp, E. coli, Salmonella* reference strain, *Pasteurella multocida ultocida, Klebsiella spp, Streptococcus spp., Histophilus somni, Shigella spp. and Pseudomonas spp.).*

Table 1: Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
P. multocida kmt1	ATCCGCTATTTACCCAGTGG GCTGTAAACGAACTCGCCAC	460	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 1 min.	72˚C 10 min.	[28]
Staph 16S rRNA	CCTATAAGACTGGGATAACTTCGGG CTTTGAGTTTCAACCTTGCGGTCG	791						[29]
Klebsiella KP16	GCAAGTCGAGCGGTAGCACAG CAGTGTGGCTGGTCATCCTCTC	260						[30]
Salmonella spvC	CGGAAATA CCATCTACAAATA -CCCAAACCCATACTTACTCTG	669						[31]
Mycoplasma spp 16S rRNA	GTTTGATCCTGGCTCAGGAYDAACG CTTGTGCGGG YYCCCGTCAA TTC	900						[32]
E. coli est	ATTTTTMTTTCTGTATTRTCTT CACCCGGTACARGCAGGATT	190						[33]

3. RESULTS

3.1. Isolation and identification

In this study, as shown in Table (2) bacteriological examination was done for 160 nasopharyngeal swabs by incidence of 59 (36.8%) bacteria isolate which were collected from 160 camels aged from 3 to 7 years suffering from pneumonia at Matrouh and Alexandria Governorates. Six genera of pathogenic bacteria were isolated from diseased camels. The frequency of isolation of these bacteria in term of abundance were as follows, klebsiella spp.,20 (12.5%), E. coli,17 (10.6%), Staphylococcus spp., 15 (9.4%), Salmonella spp., 4(2.5%) and Pasteurella multocida 3 (1.9%) to the total number of samples.

3.2. Polymerase chain reaction (PCR)

By using of Polymerase chain reaction (PCR) with species specific primers for the detection of each of the 6 genera of pathogenic bacteria, as shown in Table (2) and Fig (1-5), all bacteriologically positive samples were positive with PCR, whereas, PCR detected 23 bacteriologically negative samples. Moreover, as shown in Fig (6), PCR can success to detect Mycoplasma spp. which failed to detect by culture. The incidence of 82 (51.2%) **bacteria isolates** by PCR **were obtained** and the frequency of isolation of these bacteria were as follows, klebsiella spp., 26 (16.2%), E. coli, 21(13.1%), Staphylococcus spp., 20 (12.5%), Salmonella spp., 7 (4.3%), Pasteurella multocida 5 (3.1) and Mycoplasma spp., 3 (1.9%) , Regarding, 260bp, 190 bp, 791bp, 699bp 460bp and 900bp PCR products were observed on agarose gel electrophoresis for Klebsiella, E. coli, Staphylococcus spp., Salmonella spp., Pasteurella multocida and Mycoplasma spp. respectively. So, our result revealed that, the most common pathogens causing pneumonia in camel are *klebsiella* spp., E. coli followed by *Staphylococcus* spp. Then each primer pair was also tested using both single DNA and a mixture of the 6 DNA templates. When different combinations of the 6 bacteria (using 1 to 6 randomly selected bacteria) were used in the mPCR, the respective bacterial amplicons were produced and were separated on agarose gel electrophoresis (Fig 7).

Table 2: Comparison between culture and PCR for detection of the 6 microorganisms.

Microorganisms	Culture	PCR
klebsiella	20 (12.5%)	26 (16.2%)
E. coli	17(10.6%)	21 (13.1 %)
Staphylococcus spp.	15 (9.4%)	20 (12.5%)
Salmonella spp.	4 (2.5%)	7 (4.3%)
Pasteurella multocida	3 (1.9%)	5 (3.1%)
Mycoplasma spp	---	3 (1.9%)
Total	59 (36.8%)	82 (51.2%)

Primer species specificity Fig (1-6):

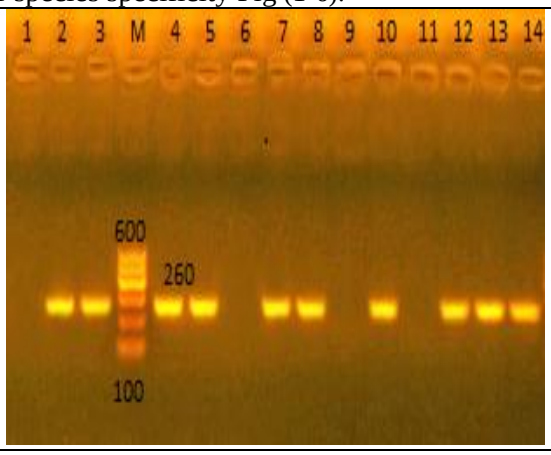

Fig 1: *klebsiella spp.*
PCR amplification *of* ***klebsiella spp***. DNA from different samples; M ,Molecular weight marker showed 100bp-600 bp DNA ladder the type code is shown above the lanes, lane 1 negative control, lane 4 positive control , lanes2,3, 5,7,8,10,12,13,14 represents the positive product of ***klebsiella spp***, lanes 6, 9,11 represents the negative.

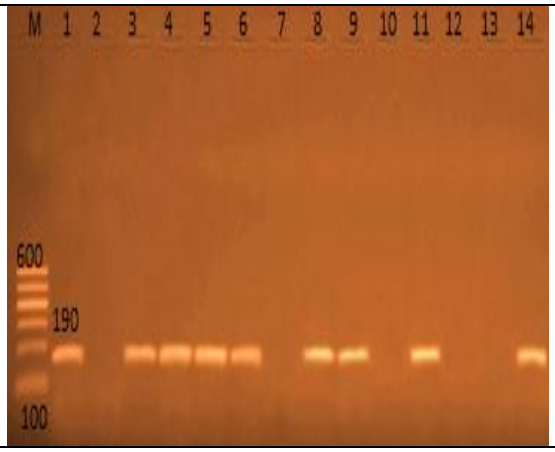

Fig 2: *E. coli.*
PCR amplification *of* ***E. coli***. DNA from different samples; M, Molecular weight marker showed 100bp-600 bp DNA ladder the type code is shown above the lanes, lane 1 positive control 190bp, lane 2 negative control , lanes3,4,5,6,8,9,11,14 represents the positive product of ***E. coli***, lanes 7, 10,12,13 represents the negative.

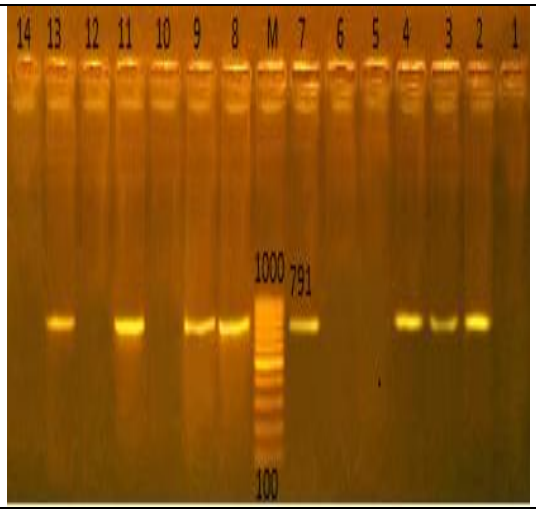

Fig 3: *Staphylococcus spp.*
PCR amplification *of Staphylococcus spp*. DNA from different samples; M ,Molecular weight marker showed 100bp-1000bp DNA ladder the type code is shown above the lanes, lane 1 negative control , lane 7 positive control 791bp, lanes2,3,4,8,9,11,13represents the positive product of *Staphylococcus spp,* lanes 5,6,10,12,14represents the negative.

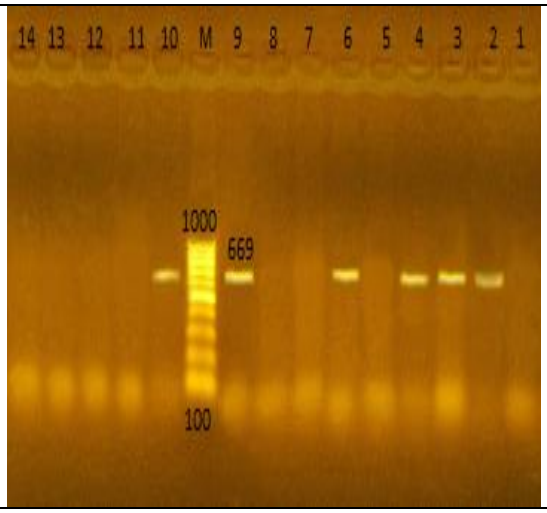

Fig 4: *Salmonella spp.*
PCR amplification *of Salmonella* ***spp***. DNA from different samples; M ,Molecular weight marker showed 100bp-1000 bp DNA ladder the type code is shown above the lanes, lane 1 negative control, lane 9 positive control ,2,3,4,6,10 represents the positive product of Salmonella ***spp***, lanes 5, 7,8,9,11,12,13,14 represents the negative.

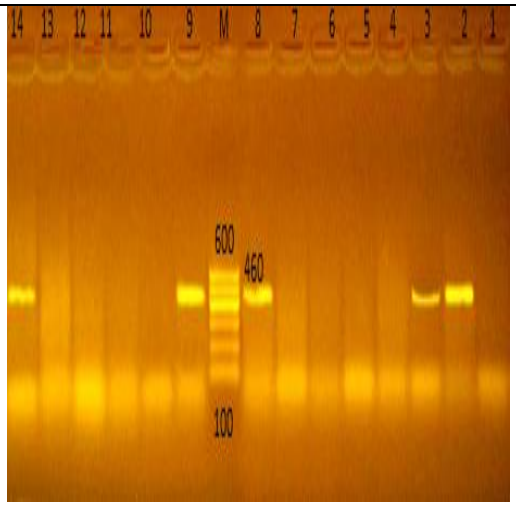

Fig 5: *Pasteurella multocida.*
Amplification of *Pasteurella Multocida* DNA from different samples; M, Molecular weight marker showed 100bp-600 bp DNA ladder The type code is shown above the lanes, lane 1 negative control, lane 8 positive control 460bp, lanes2,3, 9,14 represents the positive product of *Pasteurella multocida* lanes 4, 5,6,7,10,11,12,13 represents the negative.

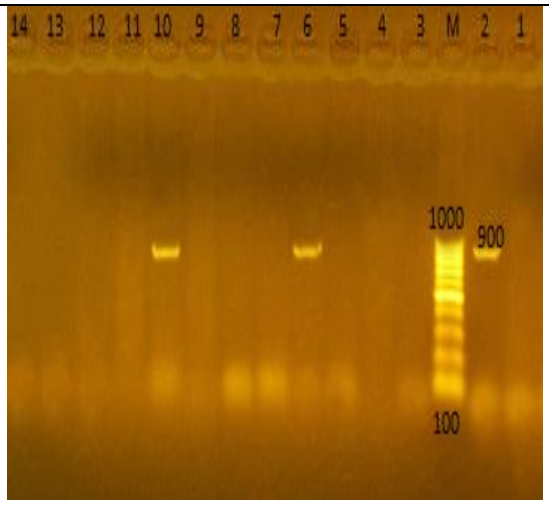

Fig 6: *Mycoplasma spp.*
PCR amplification *of* ***Mycoplasma spp*** DNA from different samples; M ,Molecular weight marker showed 100bp-1000 bp DNA ladder the type code is shown above the lanes, lane 1 negative control, lane 2 positive control, lanes6,10 represents the positive product of ***Mycoplasma spp***, lanes 3, 4,5,7,8,9,11,12,13,14 represents the negative.

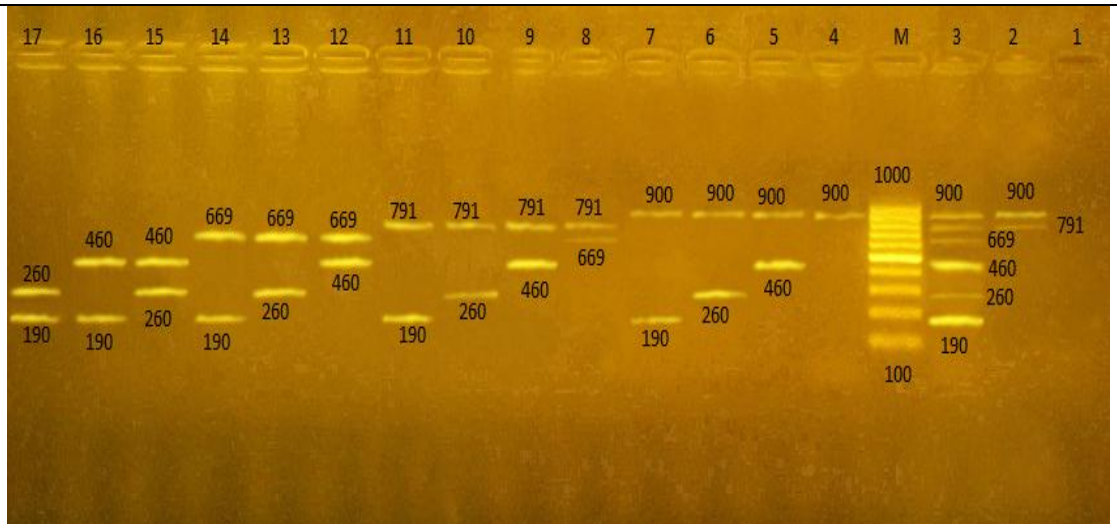

Fig 7: Agarose gel electrophoresis of multiplex PCR amplified products. M, 100-bp DNA marker; lane 1, negative control; lane 3, 190 bp for Escherichia coli; 260 for *klebsiella spp* ;460 P. multocida; 699 bp for salmonella spp791 for *Staphylococcus spp*; and 900 bp for *Mycoplasma spp*;lane2 *Mycoplasma spp* and *Staphylococcus spp*; lane 4 *Mycoplasma spp*; lane5 *Mycoplasma spp* and *Pasteurella multocida*,lane6 *Mycoplasma spp* and *klebsiella spp,* lane7 *Mycoplasma spp* and Escherichia coli ,lane8 *Staphylococcus spp and salmonella spp* lane9 *Staphylococcus spp and* Pasteurella multocida, lane10 *Staphylococcus spp and Pasteurella multocida*,lane 11 *Staphylococcus spp and* Escherichia coli, lane 12 salmonella spp *and Pasteurella multocida*, lane 13 salmonella spp and *klebsiella spp,* lane 14 *salmonella spp and Escherichia coli*, lane 15 Pasteurella multocida and *klebsiella spp,* lane 16 *Pasteurella multocida* and Escherichia coli, lane 17 *klebsiella spp and* Escherichia coli.

3.3 Multiplex PCR

Analytical Specificity of mPCR

On the current work, (Fig8) we applied analytical specificity of mPCR to other bacteria that can also be found in camels to detect the cross-reactivity between mPCR primer sets except those of *Klebsiella spp., Escherichia coli, Staphylococcus spp., Salmonella spp, Pasteurella multocida* and *Mycoplasma spp.* The result revealed that, no amplicons were produced with *Streptococcus spp., Histophilus somni, Shigella spp. and Pseudomonas spp.*

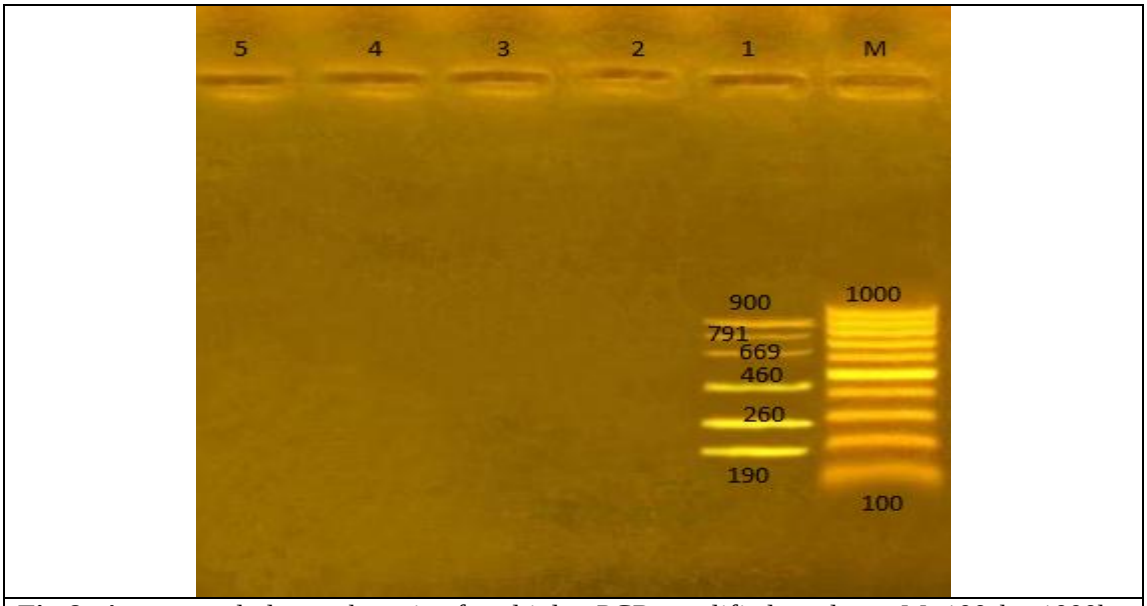

Fig 8: Agarose gel electrophoresis of multiplex PCR amplified products. M, 100-bp-1000bp DNA marker; lane 1 mixture of 190 bp for Escherichia coli; 260 for *klebsiella spp* ;*460 P. multocida*; 670 bp for *salmonella spp*791 for *Staphylococcus spp*;and 900 bp for *Mycoplasma spp*; lane 2, *Streptococcus spp*; lane 3, *Histophilus somni*; lane *4 Shigella species*; lane 5 *Pseudomonas spp* all are negative.

3.4. Application of the multiplex PCR (mPCR) for the identification of pathogens isolated from field samples (Fig. 9)

To evaluate the discriminating power of the method even further and to test its applicability, 100 nasal swabs of camels from field sample were tested for *Klebsiella, Escherichia coli, Staphylococcus spp, Salmonella spp, Pasteurella multocida* and *Mycoplasma spp.* using the and confirmed by routing PCR with the same 6 sets of primers. Multiplex PCR assays were found to be more rapid, sensitive and give a chance of covering the 6 microorganisms in a short time in one tube for accurate detection. In which the mPCR products were 260bp for *Klebsiella*, 190 bp for *E. coli*, 270 bp for *Staphylococcus* spp., 669 bp for *Salmonella* spp., 460 bp for *Pasteurella multocida* and 900 bp for *Mycoplasma spp*.

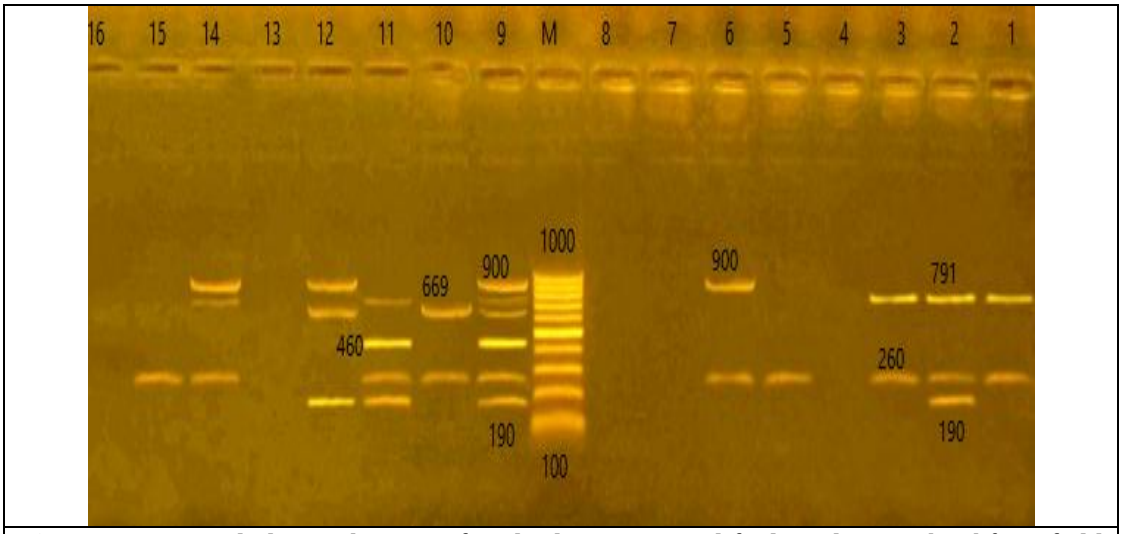

Fig 9: Agarose gel electrophoresis of multiplex PCR amplified products isolated from field samples. M, 100-bp molecular size DNA ladder (Promega); positive mixture Lane 9, lane16 negative control, **L**ane 1,3 *Staphylococcus spp* and *klebsiella spp*; *lane 2 Staphylococcus spp, klebsiella spp and Escherichia coli*,, lane 4,7,8,13 negative sample, lane 5,15 *klebsiella spp*; *lane 6 mycoplasma spp* and *klebsiella spp*; *lane 10 salmonella spp and klebsiella spp*; *lane 11* Escherichia coli, *klebsiella spp* P. multocida and *Staphylococcus spp*; *lane 12*Escherichia coli, *salmonella spp and mycoplasma spp*; *lane 14, klebsiella spp, Staphylococcus spp* and *mycoplasma spp; lane 15 klebsiella spp.*

4. DISCUSSION

Pneumonia is the main causes of morbidity and mortality, which lead to a high economic loss in camels. Generally, pneumonia was the most important symptoms and has been reported as common owner's complaints in camels at the area of investigation. The examined camels suffering from nasal discharge different from watery to abundant mucopurulent, depression, coughing, loss of appetite, fever, dyspnea, and hyperventilation.

In the present study, the result indicates that 59 (36.8%) different bacterial isolates were obtained by culture of the samples. This is confirmed by several researchers who previously isolated various bacterial species from many regions of the camel respiratory tract, nasal tracts, tonsil, trachea, and lungs, with different percentage.[34-35-36] On the otherwise, it was evident that the most common isolate caused pneumonia was *klebsiella spp., E. coli*, and followed by *Staphylococcus spp*., this nearly similar but differ in prevalence with Nahed.[37] who implicated that, *Klebsiella pneumonia* and *Staphylococcus aureus* were the most common bacterial pathogens isolated from both healthy and diseased camel. The differences in prevalence may be due to using different sample types, different methods for detection and identification. However, it was not the aim of the present work to study the incidence of different microorganisms rather than to obtain methods of early diagnoses and detection of bacterial pathogens causing pneumonia in camels.

Early diagnosis and identification of bacterial causative agents in cases of pneumonia can reduce morbidity, mortality and decrease the overuse of antimicrobials drugs.

Conventional culture methods taking a lot of time and it tedious, monotonous costly as they require hundreds of antisera as well as well trained technicians, besides, are not always accurate to detect respiratory tract pathogens and these traditional methods have low specificity and sensitivity for discovering microorganisms. These findings confirmed the result which was Saied in previous literature.[38-39-40] The other disadvantageous that culture media need many requirement and equipment for different organisms and due to many varieties of microorganisms the results are difficult to interpret. This result is approved with Chan.[41]

So, this study was designed to compare the fast-responsive PCR with conventional culture method in pneumonia and evaluate the reliability of multiplex PCR assay (mPCR) for simultaneously detecting and identifying 6 pathogenic bacteria. The result revealed that, all bacteriologically positive samples were positive with PCR whereas, PCR detect 23 bacteriologically negative samples.

On the other hand, **59 (**36.8%**)** and 82(51.2%) bacterial isolates which include *Klebsiella spp*.,20 (12.5%) and 26 (16.2%), *E. coli*,17 (10.6%) and 21 (13.1%), Staphylococcus spp.,15 (9.4%) and 20 (12.5%), *Salmonella spp*.4 (2.5%) and 7 (4.3%) and *Pasteurella multocida* 3 (1.9%) and 5 (3.1%) were obtained by culture and PCR respectively. Also, as seen in this work, a reference to mycoplasma there was no positive result obtained by culture method, but 3(1.9%) of nasal swabs were positive by using PCR method. This means that, species specific PCR more accurate, sensitive and specific method for detecting of various pathogenic bacteria and PCR due to no need of purification and cloning, pathogenic bacteria are easy to detected even within the mixed organisms in a sample, without spending long times. This finding was consistent with many previous reports.[42]

In the present work, culture failed to detect mycoplasma spp. but PCR succeeded to detect it. This is going in hand with Alaa.[43] who showed that, by using culture method, *Mycoplasma* was not isolated from any of these samples in a PPLO broth and/or solid culture media. Moreover, Mycoplasma is difficult to be cultured and it is often diagnosis usually depend on serology in the past.[44] However, serology is not sufficiently rapid and reliable especially in specificity and is usually give its positive result after 7 days after the onset of disease.[41-42-45] Regarding, the pathogen cannot be identified during the period of incubation and the serological cross-reactions among the Mycoplasma spp. are causing the serious problem.[13]

In this work, the rates of PCR 82(51.2%) was higher than obtained by culture **59 (**36.8%**)**, the reasons for this might due to the selectivity of culture conditions inhibition the growth of multiple species of bacteria. This similar with Templeton.[46] Who reported higher pathogen identification rates with the PCR method. Also, **Mustafa**[47] deal with pneumonia cases, microbial agents were detected in 39.1% and 65.2% by culture and PCR respectively. Besides, **Aydemir.**[48] conducted that bacterial isolate was detected in 62 (31.5%) by culture while in PCR method was detected in 125 cases (63.5%).

Multiplex PCR is effective and powerful tool in clinical microbiology and recently has been widely used to detect bacteria and genes of interest also, from it's advantage it saving time, less labor and decrease the risk of manipulation with pathogenic microorganisms for the long time, Multiplex PCR assay provides sensitive and reliable results and allows for the cost-effective detection of more than one bacterial pathogens in single reaction tube.[49-50-51-52]

In our result, by using species specific PCR and multiplex PCR, help us to detected and identified Mycolpasma spp. and this finding was consistent with many previous reports.[11-43-53]

The mPCR primer pairs must be species specific to the genes of interest, and the PCR products must be of different sizes.[54] (Fig. 1-6), (Fig.7).

The use of mPCR in the present study (Fig.9) based on detection of DNA revealed detection and identifying of 6 organisms based on detection of KP16, est, 16S rRNA, spvC, kmt1 and 16S rRNA genes which responsible for attachment of each of *Klebsiella, E. coli, Staphylococcus spp., Salmonella, P. multocida* and *Mycoplasma spp*., respectively and samples positive for all of these bacterial pathogens produced clear agarose gel bands.

Also, as shown in (Fig.8) no cross-amplification of the 6 targets was observed with the DNA from other pathogens (*Streptococcus spp., Histophilus somni, Shigella species and Pseudomonas spp*.). Besides, mPCR results showed that, the field samples were positive for one or more of these organisms which was same result obtained by single PCR, confirm that, the mPCR was a rapid and cost-effective diagnostic tool to survey these 6 bacteria pathogens in camels at the same time in one tube, and the performance of mPCR infield further validates its use as an optimal tool for the 6 bacterial pathogens. Also, it is sensitive enough to apply for clinical evaluations. This is in agreement with other authors.[44-45]

A novel protocol was developed to simultaneously detect and identify P. multocida, Salmonella, and E. coli, which are the most important bacterial pathogens, based on the problem that similar clinical signs and coinfections with 2 or more than 2 pathogens always occur in camels. Numerous PCR tests have been described for detecting each of these 6 bacterial pathogens, separately and there is information are available for detecting of these 6 pathogens together in a single tube.[43-52] Our investigation showed that, mPCR assay was a rapid and cost-effective than uniplex and this is agreement with Hamido[52] who methane that, in a uniplex PCR Potential problem include false negatives due to reaction failure or false positives due to contamination. On the other hand, mPCR has high efficiency in which the expense of reagents and preparation time is less in mPCR than in systems where several tubes of uniplex PCRs are used, also multiplex reaction is ideal for conserving costly polymerase and templates in short supply. Beside, the quality of the template determined more effectively in multiplex than in a uniplex PCR reaction and the exponential

amplification and internal standards of multiplex PCR can be used to assess the amount of a particular template in a sample.

Confirming a previous study that, camels can be infected with 2 or more bacterial pathogens simultaneously. Coinfection with 2 or more pathogens increases the risk of being unable to differentiate the diseases based on clinical signs.[57] These many infections are difficult to diagnose Muna and Musa.[58] So, the mPCR can detect many bacterial microorganisms at the same time in comparison with the uniplex PCR which detect only one bacterial microorganism, Thus, mPCR is more rapid, cost-effective, provide more useful for clinical diagnosis and epidemiological surveillance, the same result was recorded by.[51]

5. CONCLUSION

The multiplex PCR method is high in performance and is senior in the detection of multiple pathogens at the same time and also give rapid identification of bacteria and causative agents of infection. Therefore, we recommended that, the widespread use of methods will provide the success of rapid diagnoses and treatments of respiratory infection in camels with highly specific antibiotic.

REFERENCES

1. Abdoun KA, Alsofi MA, Samara EM, Alhidary LA, Okab AB. Evaluation of the effects of chromium supplementation on growth and nitrogen balance of camel calves under summer conditions. Tropical Animal Health and Production, 2015; 619–621.
2. Muhammad YAQOOB, Haq NAWAZ. Potential of Pakistani camel for dairy and other uses 2007; Animal Science J, 78: 467–475.
3. AHMAD S, YAQOOB M, HASHMI N, AHMAD S, ZAMAN MA, TARIQ M. Economic Importance of Camel A Unique Alternative under Crisis 2010; Pak Vet J., 30(4): 191-197.
4. Kebeda F, Gelaye E. Studies on major respiratory diseases of camel (*Camelus dromedarius*) in Northeastern Ethiopia 2010; Afr. J. Microbiol. Res, 4: 1560-1564.
5. Funk AL, Goutard FL, Miguel E, Bourgarel M, Chevalier V, Faye B, Peiris JS,etal. MERS-CoV at the Animal-Human Interface: Inputs on Exposure Pathways from an Expert-Opinion Elicitation 2016; Frontiers in Vet. Science, 3: 88.
6. Ali MA, Shehata MM, Gomaa MR, Kandeil A, El-Shesheny R, Kayed AS Systematic, active surveillance for Middle East respiratory syndrome coronavirus in camels in Egypt 2017; Emerging Microbes and Infections, 6(1).
7. Hassan AKM, Mustafa AA Isolation of Pasteurella multocida type B from an outbreak of haemorrhagic septicemia in camels in Sudan 1985; Revue D'Elevage et de Medicine Veterinaire des Pays Tropicaux, 38: 1 31–33.
8. Wernery U, Kaaden OR Infectious Diseases of Camelids 2002; 2nd Edition. Blackwell Wissenschafts Verlag, Berlin, Germany pp. 110.
9. Mohamed RA, Abdelsalam E B A review on pneumonic pasteurellosis (respiratory mannheimiosis) with emphasis on pathogenesis, virulence mechanisms and predisposing factors. Bulg 2002; J. Vet. Med., 11(3): 139−160.
10. Brisse S, Duijkeren VE Identification and antimicrobial susceptibility of 100 Klebsiella animal clinical isolates 2005; Vet. Microbiol, 105: 307-312.
11. Sharma SK, Mudgal NK, Sharma P, Shrngi BN Comparison of phenotypic characteristics and virulence traits of Klebsiella pneumoniae obtained from pneumonic and healthy camels (Camelus dromedarius) 2015; Adv. Anim. Vet. Sci., 3(2): 116-122. https://doi.org/10.14737/journal.aavs/2015/3.2.116.122.
12. McAuliffe L, Brando K, Roger DA, Robin AJN. Molecular epidemiological analysis of Mycoplasma bovis isolatd from the United Kingdom show two genetically distinct cluster 2014; J Clin Microbiol, 42(10): 4556-4565.
13. Bashiruddin JB, Frey J, Konigsson MH, Hotzel H and Sachse K. Evaluation of PCRsystems for the identification and differentiation of Mycoplasma agalactiae and Mycoplasma bovis: a collaborative trial 2005; Vet J, 169(2): 268-275.
14. Svensson C, Jensen MB. Short communication: Identification of diseased calves by use of data from automatic milk feeders 2007; J Dairy Sci, 90(2): 994-997.
15. Abubaker M, Nayel, MN, Fadlalla MF, Abdel Rhma AO. The incidence of bacterial infections in young camels with reference to E. coli. The International Scientific Conference on Camels. May 10-12-2006; Qassim University–College of Agri. and Vet. Medicine, pp. 479–482.
16. Fouda TA, Al Mujalii AM. Pneumo-enteritis in Arabian camel-calves (Camelus dromedarius): clinical and laboratory investigations 2007; J. Camel Pract. Res., 14(2): 119–124.
17. Delgado MO, J.Timenetsky. Immunoblot profiles of sera from laboratory rats naturally infected with Mycoplasma pulmonis and technicians exposed to infected animal facilities 2001; Braz. J. Microbiol, 32(4): 301-304.
18. Mbuthia PG, Njagi LW, Nyaga PN, Bebora LC, Minga U, Kamundia J.etal Pasteurella multocida in scavenging family chickens and ducks: carrier status, age susceptibility and transmission between species 2008; Avian Pathol, 37: 51-57.
19. Ilona V. Gluecks, Astrid Bethe , Mario Younan, Christa Ewers. Molecular study on Pasteurella multocida and Mannheimia granulomatis from Kenyan Camels (Camelus dromedarius) 2017; BMC Vet. Research, 13: 265.

20. Townsend KM, Boyce J D, Chung JY, Frost A J, Adler B. Genetic organization of Pasteurella multocida cap loci and development of a multiplex capsular PCR typing system 2001; J. Clin. Microbiol, 39: 924–929.
21. Kardos G J, Nagy M , Antal A, Bistyak M, Tenk, Kiss I. Development of a novel PCR assay specific for Riemerella 2007; anatipestifer. Lett. Appl. Microbiol, 44: 145-148.
22. Kim J, Demeke T, Clear RM, Patrick SK .Simultaneous detection by PCR of Escherichia coli, Listeria mono cytogenes and Salmonella typhimurium in artificially inoculated wheat grain 2006; Int. J. Food Microbiol, 111: 21-25.
23. Hu, Q. H., J. Tu, X. G. Han, Y. Y. Zhu, C. Ding, SQ. Yu. Development of multiplex PCR assay for rapid detection of Rie- merella anatipestifer, Escherichia coli, and Salmonella enterica simultaneously from ducks. 2011; J. Microbiol. Methods, 87: 64-69.
24. Rosendal S. Ovine and caprine mycoplasmas.in Mycoplasmosis in animals ": laboratory diagnosis. Edited by Whitford, H.W.; Rosenbusch: R. F. and Lauerman, L. H lowa. 1994; state university press, Ames, PP. 84-95.
25. Mona A. Mahmoud, Wassif I M, El-Sayed AA, Awad WSA, Heba S Farag , Eman A. Noaman. MOLECULAR IDENTIFICATION OF RESPIRATORY BOVINE MYCOPLASMA ISOLATED FROM ARABIAN CAMELS IN EGYPT 2017; j.Egypt. vet. med. Assoc, 77(4): 919–936.
26. Quinn PJ, Carter ME, Markey B, Carter GR. Clinical Veterinary Microbiology. 5th Edn. Harcourt Publishers Ltd., 1999; ISBN: 0-7234-17113.
27. Holth IG, Krieg NR, Sneath PHA, Staley IT, Williams ST. Bergey's Manuel of Determinative Bacteriology.9th Edn. Lippincott Williams and Wilkins, Philadelphia. 2000; ISBN: 0-683-00603-7.
28. OIE. OIE *Terrestrial Manual* 2012; CHAPTER 2. 4. 12. HAEMORRHAGIC SEPTICAEMIA.
29. MASON WJ, BLEVINS JS , BEENKEN K, WIBOWO N, OJHA N, SMELTZER MS. Multiplex PCR Protocol for the Diagnosis of Staphylococcal Infection.2001; J OF CLIN. MICROBIOLOGY, 39(9): 3332–3338.
30. Tayebeh1 F, Amani J, Nazarian S, Moradyar M, Mirhosseini SA. Molecular Diagnosis of Clinically Isolated *Klebsiella pneumoniae* Strains by PCR-ELISA. 2016; *J of Applied Biotechnology Reports, Autumn, 3(4): 501-505*
31. Swamy SC, Barnhart HM, Lee MD, Dreesen DW. Virulence determinants invA and spvC in salmonellae isolated from poultry products, wastewater, and human sources.1996; Appl Environ Microbiol. Oct, 62(10): 3768-71.
32. Pettersson B, Leitner T, Ronaghi M, Bölske G, Uhlen M,Johansson KE. Phylogeny of the Mycoplasma mycoides cluster as determined by sequence analysis of the 16S rRNA genes from the two rRNA operons. 1996; J Bacteriol, 178(14): 4131-42.
33. Aranda KR, Fabbricotti SH, Fagundes-Neto U, Scaletsky I.C. Single multiplex assay to identifysimultaneously enteropathogenic, enteroaggregative, enterotoxigenic, enteroinvasive and Shiga toxin-producing Escherichia coli strains in Brazilian children. 2007; FEMS Microbiol Lett, 267; 145–150.
34. Radostits OM, Gay CC, Hinchcliff KW, Constable PD .2007; Veterinary Medicine: A text Book of the diseases of cattle, sheep, pigs, Goats and Horses, 10 th ed. Saunders Company, Philadelphia, U.S.A., pp. 471-54.
35. Ismail M, El-Deen NE , El-Hariri M. Bacteriological Examination of Respiratory Tract of Apparently Healthy Camels in Egypt.2014; International J. of Microbiological Research, 5(1): 65–68.
36. Wareth G, Murugaiyan J, Khater DF, Moustafa SA. Subclinical pulmonary pathogenic infection in camels slaughtered in Cairo .2014; Egypt, J. of Infection in Developing Countries, 8(7): 909–913.
37. Nahed SS, Tarek R A E, Amani A, Hafez, Iman AE, Asmaa A D. Clinicopathological and Bacteriological Studies on Pneumonia in Camel (Camelus dromedarius).2016; J. of Vet. Advances, 6(4): 1228-1236.
38. San Pedro GS, Campbell GD Jr. Limitations of diagnostic testing in the initial management of patients with community-acquired pneumonia. 1997; Semin Respir Infect.; 12(4): 300-307.
39. Centers of Disease Control and Prevention; CDC2003. Salmonella .Annual Summary 2002 Centers for Disease control and Prevention, Atlanta, pp.106-115.
40. Musher DM, Roig IL, Cazares G, Stager CE, Logan N, Safar H. Can an etiologic agent be identified in adults who are hospitalized for community-acquired pneumonia: results of a one-year study. 2013; J Infect., 67(1): 11-18. doi: 10.1016/j. jinf.2013.03.003.
41. Chan YR, Morris A. Molecular diagnostic methods in pneumonia.2007; Curr Opin Infect Dis., 20: 157-64.
42. Schott II, Ewart SL, Walker R.D, Dwyer RM, Eberhart SW. An outbreak of salmonellosis among horses at a veterinary teaching hospital.2001; J. Am. Vet. Med. Asso., 218: 1152-1159.
43. Alaa A. Mohamed, Magdy H. Yassin, Mohamed M. Hassan, Ayman M. Sabry, Amena M. Ibrahim. Molecular and Bacteriological Diagnosis of Mycoplasma Species Infection in Camels at Taif Governorate, Saudi Arabia. 2018; ARRB. Annual Research & Review in Biology, 23(5): 1-6.
44. Tjhie JH, van Kuppeveld FJ, Roosendaal R, Melchers WJ, Gordijn R, MacLaren DM, et al. Direct PCR enables detection of Mycoplasma pneumoniae in patients with respiratory tract infections. 1994; J Clin Microbiol, 32: 11-6.

45. Serpil KAHYA , Kaan ÖNAT , Evren ERKÖSE , Seran TEMELLİ , Aysegul EYİGOR , Kamil Tayfun CARLI. Effect of Mentofin application on the clearance of Mycoplasma gallisepticum (MG) from naturally infected layer chickens' trachea 2015; Ankara Üniv Vet Fak Derg, 62: 17-21.
46. Templeton KE, Scheltinga SA, Eeden VD, Graffelman WC. Improved diagnosis of the etiology of community- acquired pneumonia with real-time polymerase chain reaction 2015; Clin Infect Dis., 41(3): 345–351.
47. Mustafa MIA, Marzoog F, How SH, Kuan YC. The use of multiplex real time PCR improves the detection of the bacterial etiology of community acquired pneumonia 2011; Tropical Biomed, 28(3): 531-544.
48. Aydemir O, Aydemir Y, Ozdemir M. The role of multiplex PCR test in identification of bacterial pathogens in lower respiratory tract infections. Pak J Med Sci, 2014; 30(5): 1011-1016.
49. Kong R Y, Lee S K Y, Law TW, Law S H, Wu R S. Rapid detection of six types of bacterial pathogens in marine waters by multiplex PCR.2002; Water Res., 36: 2802–2812.
50. Abeer, A. AlAll, Azza S.A. Gouda, Dardir HA, Ibrahim AK. Prevalence of Some Milk Borne Bacterial Pathogens Threatening Camel Milk Consumers in Egypt, 2012; Global Veterinaria, 8(1): 76-82.
51. Abdoun KA, Alsofi MA, Samara EM, Alhidary LA, Okab AB, Evaluation of the effects of chromium supplementation on growth and nitrogen balance of camel calves under summer conditions, 2015; Tropical Animal Health and Production, 47: 619–621.
52. Using Uniplex and Multiplex Polymerase Chain Reaction Assays For Molecular Diagnosis Of Indicator Pathogens in Non Sterile Pharmaceutical Products Hamido M. Hefny and Mohammed S. Alhussaini Life Science Journal, 2015; 12(11).
53. Tarek R. Abo-Elnaga, Azza S.A. Gouda, Yasser M. Kamel, Mona A. Mahmoud , Wafaa A. Osman . Application of the Polymerase Chain Reaction for Identification of Mycoplasma Isolated from Aborted She Camel 2012; Global Veterinaria, 8(4): 386-392.
54. Chang H K, Park J H, Song MS, Oh TK, Kim S. Y, Kim C. J,etal detection and subtyping of influenza type A viruses from clinical specimens, 2008; J. Microbiol. Biotechnol, 18: 1164–1169.
55. Nguyen, T. T. T., T. K. N. Nguyen, V. T. Phan, T. T. Tran, M. J. Cardosa, P. C. McMinn, and P. Phuektes. 2010. Development of a multiplex polymerase chain reaction assay for simultaneous identification of human enterovirus 71 and coxsackievirus A16. J. Virol. Methods, 170: 134–139.
56. Liu, S. S., Y. R. Zhao, Q. B. Hu, C. C. Lv, C. F. Zhang, R. Zhao, F. Hu, W. C. Lin, and S. J. Cui. 2011. A multiplex RT-PCR for rapid and simultaneous detection of porcine teschovirus, classical swine fever virus, and porcine reproductive and respiratory syn- drome virus in clinical specimens. J. Virol. Methods, 172: 88–92.
57. Y.H. Al-Tarazi, 2001.Bacteriological and Pathological Study on Pneumonia in the One-Humped Camel (Camelus dromedarius) in Jordan, Revue Élev. Méd. vét. Pays trop., 2001; 54(2): 93-97.
58. Muna E. Ahmed and Musa Tibin Musa. Characterization of Bacteria Isolated from Dromedary Camels Affected with Pneumonia for the First Time in Sudan Annual Research & Review in Biology, 2015; 7(1): 61-67.
59. Benson R, Tondella ML, Bhatnagar J, Carvalho MDG S, Sampson JS, Talkington D F, et al. Development and evaluation of a novel multiplex PCR technology for molecular differential detection of bacterial respiratory disease pathogens. J. Clin. Microbiol, 2008; 46: 2074–2077.