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Prevalence of some bacterial pathogens causing calf pneumonia with special reference to *Mannheimia haemolytica*

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Abstract

Calf pneumonia is considered one of the most common and serious problems affecting calves and younger cattle all over the world causing significant economic losses due to high morbidity and mortality rate. A total of 120 samples (nasal swabs and lung tissues) were collected from calves suffering from respiratory manifestations and apparently healthy calves from different farms and small holders at El- Beheira governorate and examined bacteriologically. From diseased calves, there were 30 and 25 positive samples from nasal swabs and lung tissue; respectively. Concerning apparently healthy calves, there were 9 positive nasal swab samples. The prevalence of *M. haemolytica*, *Pseudomonas spp*, *S. aureus and E. coli* in the examined samples was *5(*4.16%), 8(6.66 %), 21(17.5 %) and 30(*25* %); respectively. Antibiogram of all isolated bacteria was studied and discussed. *E. coli* isolates were serogrouped into O_{143}, O_{119}, O_{63} and O_{128}. Molecular characterization was carried out for confirmation of the isolates as well as detection of some virulence genes and antibiotic resistance genes in *M. haemolytica* isolates.

Key words: calves pneumonia, bacterial pathogens, *Mannheimia haemolytica*, antibiotic resistance genes and virulence genes

Introduction:

Bovine Respiratory Disease (BRD) is one of the most important diseases causing enormous economic losses in veal farms. It develops due to stressors such as weaning, transport, pooling of cattle from multiple sources, dusty conditions, parasitism, co-occurring diseases, and extreme weather Lee et al. (2020). *M. haemolytica* and *P. multocida* remain the most common bacterial pathogens associated with pneumonia in cattle in addition to numerous bacteria such as Mycoplasma, *E. coli*, *Staphylococcus* spp., *Streptococcus* spp., *P. aeruginosa*, *Proteus* spp., *Corynebacteria* and, *K. pneumoniae* Asaye et al. (2015).

M. haemolytica is an opportunistic pathogen that has been detected residing in the respiratory tract of healthy and sick ruminants and capable of causing infection in cases of compromised body defense by a variety of stress factors such as transportation, malnutrition, adverse physical, environmental or climatic conditions Pardon et al. (2020).

Pneumonic pasteurellosis due to *M. haemolytica* is one of the most important disease causing economic losses in the livestock industry. It is one of the main causes of mortality in calves' farms worldwide mainly manifested within 4 weeks of weaning when the calves are sorted and distributed to different farms. It is given a common surname "Shipping Fever" Ali and Al Balaa (2019). *M. haemolytica* has many types of virulence factors that promote its adhesion, colonization and proliferation within the respiratory tract Klima et al. (2014).

Therefore, this study aimed to isolation of some bacterial pathogens causing *pneumonia* and death in *calves* from different farms and small holders at El- Beheira governorate with special reference to *M. haemolytica*, application of PCR for confirmation of the isolates as well as detection of some virulence genes and antibiotic resistance genes. The antimicrobial susceptibility testing of the isolates were studied and discussed.

Materials and Methods:

Ethical approval. This study was conducted at the Animal Health Research Institute, Dokki, Giza, Egypt and the protocol approved by the Institutional Animal Care and Use Committee, Agriculture Research Center. Giza, Egypt.

Samples collection:

A total Number of 120 samples were collected from clinically diseased calves suffering from respiratory manifestation including 60 deep nasal swabs, 40 lung tissue from (freshly dead

and emergency slaughtered calves) and 20 deep nasal swabs from contact apparently healthy animals from some farms and small holders in different localities of El-Beheira governorate Table (2). Samples were collected under aseptic conditions and transferred as soon as possible in ice box to laboratory for bacteriological examination.

Table (1): Type and number of examined calves samples:

Animal	Type of samples	No of examined samples
Diseased calves	Nasal swabs	60
	Lung tissue	40
Apparently healthy calves	Nasal swabs	20

Bacteriological Examination and Antibiogram of the isolated bacterial pathogens:

The collected samples were prepared and examined bacteriologically according to Quinn et al. (2011).

Serogrouping of *E. coli* isolates: it was performed using slide agglutination test using polyvalent and monovalent *E. coli* antisera (Denka Seiken Co. LTD, Tokyo, Japan for antisera**)** according to (Quinn et al. 2011).

Antimicrobial susceptibility testing was performed according to CLSI (2022) using disc diffusion method on Muller Hinton agar using available antibiotic discs including Levofloxacin (5µg) , Amikacin (30µg), Gentamycin (5µg) , Enrofloxacin (5µg), Amoxicillin (25µg), Oxytetracyclin (30µg), kanamycin (30µg), Cefotaxime (30µg), Florphenicol (30µg), Cefoquinome (30µg), Penicillin G (10µg) and sulphamethoxazol-trimethoprim (25µg).

Molecular characterization (PCR) of isolates:

DNA was extracted from suspect isolates using the QIAamp DNA Mini Kit (Qiagen, Germany, GmbH). The primers used were supplied by Metabion (Germany) and are listed in Table (1). PCR amplification with EmeraldAmp Max PCR Master Mix (Takara, Japan) . The reaction was performed in an Applied Biosystem 2720 thermal cycler. The products of the PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1 × TBE buffer at room temperature using gradients of 5 V/cm and the gel was analyzed by a gel

documentation system (Alpha Innotech, Biometra) were photographed and the data analyzed by computer software.

Results

The bacteriological examination of the collected samples: in diseased calves, out of examined 60 nasal swabs and 40 lung tissues there were 30 and 25 positive samples, respectively. In addition, in apparently health calves, out of examined 20 nasal swabs there were 9 positive samples as shown in table.3.

The prevalence of different types of bacterial pathogens among examined samples: in diseased calves, out of examined 60 nasal swabs, *M. heamolytica*, *Pseudomonas spp*., *S. aureus* and *E. coli* were isolated as 2,5,9 and 14; respectively, in addition, out of examined 40 lung tissue there were 3, 3,8 and 11; respectively. Concerning apparently health calves, out of examined 20 nasal swabs, *S. aureus* and *E. coli* were isolated as 4 and 5; respectively while *M. heamolytica* and *Pseudomonas spp*. were not detected as shown in table 4.

The frequency of the mixed isolates in the examined samples:

M. heamolytica was isolated with *S. aureus* and *E. coli* from two infected calves. In addition, *M. heamolytica* was mixed with *E. coli* in one infected calve and mixed with *S. aureus* in only one case. Both *pseudomonas spp*. and *S. aureus* and both *S. aureus* and *E. coli* was mixed at a rate of 4 and 10, respectively as shown in table 5.

Results of serogrouping of *E. coli* isolates: all of them were belonged to O serogroup as, O_{143}, O_{119}, O_{63} and O_{128} as 2, 1, 1 and 1; respectively as shown in table 6.

Results of antibiogram of the isolated different types of bacteria:

M. heamolytica showed resistance to Oxyteracycline, Kanamycin, Sulphatrimethoprim, Amikacin, Amoxicillin and Penicillin G. and on the other hand showed Sensitivity to Levofloxacin, Enrofloxacin, Cefoquinome and Florphenical. In addition, *E. coli* isolates showed resistance to Kanamycin, Amoxicillin, oxytertracycline, Sulphatrimethoprim and Penicillin G, and showed sensitivity to Levofloxacin, Enrofolxacin, Gentamycin and Florphenical. *pseudomonas spp.* isolates showed resistance to Oxytetracycline, Kanamycin, Amoxicillin, Sulphatrimethoprim. and Penicillin G, and showed sensitivity to Levofloxacin,. In addition, S. aureus isolates showed resistance to kanamycin , Amikacin, Oxytetracyclin, and Penicillin G. In

the contrary, it showed sensitivity to Levofloxacin, Enrofloxacin, and Cefoquinome as shown in table 7.

Results of molecular characterization of *M. haemolytica* isolates, virulence and resistance genes:

All examined 5 *M. haemolytica* isolates showed the amplification of (ssa) gene products at 325 bp as shown in table 8 and Fig. 1. In addition, Fig. 2 &3 showed Agarose gel electrophoresis of PCR products of M. haemolytica resistance genes (tetH, blaROB1 and aphA1) gene products at 1076 bp, 685 and 489 bp respectively, and revealed that all 3 genes detected in all examined (5) isolates indicating that the isolates were highly resistant to antibiotic and illustrated the obtained resistances against tetracyclines, (tetH) penicillins (blaROB1) and aminoglycosides (aphA1) gene.

Results of molecular characterization of *E. coli, Pseudomonas spp*. isolates. Fig.6 showed that, all examined 4 *pseudomonas spp*. isolates showed the amplification of confirmatory gene product 16S rDNA at 618 bp. In addition, all examined 4 *E. coli* isolates showed the amplification of E. coli phoA confirmatory gene product at 720 bp.

Discussion

Bovine respiratory disease (BRD) is one of the major problems in the livestock industry, resulting in poor health, high mortality in young calves, reduced body weight, compromised animal welfare, and increased treatment and vaccination costs in affected herds. In addition to increased morbidity and mortality in infected calf herds requiring massive use of antimicrobial compounds, leading to the emergence of antibiotic resistance Al gammal et al. (2020)

Results in **Table 4** were nearly similar to those obtained by El Dokmak et al. (2015) who isolated *M. haemolytica* with percentage of 5 % from diseased cattle. Lower prevalence was obtained by Lasisi et al. (2016) in Nigeria who isolated *M. haemolytica* with percentage 1.22% from unhealthy lung tissue sample of cattle. In addition, Al gammal et al. (2020) isolated *M. haemolytica with* percentage of (0.6%) from all exained samples of pneumonic calves. Higher isolation rate was detected by Zaki et al. (2002), Ahmed et al. (2015), Abera et al. (2014) and El-Seedy et al. (2020) who isolated *P. haemolytica as* 8.8, 20 , 46.4, 8.4 percentage; respectively from pneumonic calves. The variation of the isolation rate of *M. haemolytica* may be due its

delicate and very sensitive character and special growth condition (Smith and Phillips 1990). On the other hand, in this study *M. haemolytica* could not be isolated from nasal swab of apparently healthy animals and this supported by Abera et al. (2014) , El-Dokmak et al. (2015) and Ahmed et al. (2017) who could not isolated *M. haemolytica* from nasal swab of apparently healthy animals. The difference in results may be due to the fact that the etiology of pneumonia is complex and multifactorial, and may be due to different isolation techniques, misidentification, environmental husbandry, seasonal variation and breed differences, laboratory facilities, different hygiene practices, different calf rearing systems, and stressors on different farms and places Hashem et al. (2022). The total prevalence of *E. coli* among the examined samples was higher than obtained by Al- gammal et al. (2020), El-Seedy et al. (2020) and Hashem et al. (2022) from diseased calves as 23, 4.2 and 5%; respectively. High isolation rate in *E. coli* and *S. aureus* in this study agree with Hafez and Yousef (2002), Sayed et al. (2002) and Algammal et al. (2020) in Egypt who recorded that beside *P. multocida, M. haemolytica, S. aureus* and *E. coli* were the most prevalent bacteria from calves with respiratory manifestations. Lower *S. aureus* isolation rate was obtained by Hashem et al. (2022) as 5%. The total prevalence of *pseudomonas spp* among the examined samples was nearly similar to Al gammal et al. (2020) who isolate *pseudomonas aeruginosa by* 5.9 % from nasal swabs from pneumonic calves and higher than obtained by El-Seedy et al. (2020) as 2.7 % *P. aeruginosa from* calves with respiratory manifestations and lower than Sedeek and Thabet (2001) as 11 % from pneumonic cattle at Assiut Governorate. In addition, Ayyoub et al. (2019) reported that *Pseudomonas aeruginosa* was isolated from beef farm suffering from respiratory signs with percentage of 15%.

This variation in the isolation rate may be attributed to different hygiene measures, operational management and stress levels. Calves can inhale many environmental bacteria and detected in both the upper and lower airways, so their opportunistic role should be investigated Al gammal et al. (2020). In addition, the negative bacterial isolation was not necessarily meaning the absence of bacterial infections but it may be attributed to that some microorganisms require specific, enriched culture media or tissue culture including *Mycoplasma* spp.; *M. bovis, Histophilus somnus* and chlamydia which are also incriminated in BRD affecting young calves

Mixed bacterial infection as shown in table 5 may be attributed to the presence of some bacteria as a normal flora on the skin and oropharynx which may be flourished causing diseases as a result of several hygienic and environmental measures or suppressed host's immune system

which damage the lining of the respiratory tract which enable these pathogens to progress deeper into the respiratory tract and cause disease El-Seedy et al. (2020).

E. coli serogroups detected in this study as shown in table 6 were similar to Abdel Hamid and Ibrahim (2017) and Al gammal et al. (2020) who detected similar *E. coli* serogroups in pneumonic and apparently health calves in Egypt. In addition, Fouad et al. (2021) detected similar *E. coli* serogroups in diarrheic calves in Egypt. The antibiogram of the isolated *M. haemolytica* in this study as shown in table 7 was similar to El-Seedy et al. (2020) who reported that *M. haemolytica isolates were* sensitive to enrofloxacin, cefiquinome, levofloxacin, ciprofloxacin and ceftriaxone in a percentage of 90, 80, 80, 70 and 70, respectively and high resistances were showed against oxytetracycline, kanamycin, amikacin and amoxicillin-calvulanic acid as 100, 90, 70 and 60%, respectively. On the other hand the results disagreed with Abera et al. (2014) in Western Ethiopia, who found that the isolates *P. multocida* and *M. haemolytica* were susceptible to most of the antibiotic discs used including amoxicillin, chloramphenicol, cephalexin, kanamycin and florfenicol. However, moderate resistance was observed to tetracycline, erythromycin and penicillin-G. Concerning the antimicrobial susceptibility testing of *S. aureus*, *E. coli* and *pseudomonas spp*. our results agreed with Kroemer et al. (2012) who suggested that enrofloxacin and norfloxacin can still be utilized with a high chance of curative success for the treatment of respiratory diseases in bovines. Algammal et al. (2020) reported that the most predominant *E. coli*, *S. aureus*, and *P. aeruginosa* strains were highly sensitive to enrofloxacin and norfloxacin and showed variable degrees of resistance against the tested antimicrobial agents. El-Seedy et al. (2020) reported that the tested isolates were sensitive to cefiquinome, levofloxacin, ciprofloxacin and enrofloxacin. On the other hand resistant to oxytetracycline and kanamycin. The multidrug-resistance was observed among the majority of the isolates against antimicrobial agents could be attributed to the random use of antibiotics over time in animal production as mentioned by Enany et al. (2019) and Algammal et al. (2020). Results in Table 8 and Fig. 1 were similar to Ayalew et al. (2013) who previously identified *M. haemolytica* OMPs that may be an important immunogen, including serotype 1-specific antigen (ssa1) by using immunoproteomic analyses and Klima et al. (2018) who recorded OMPs serine protease encoding (ssa) gene as one of the top ten antigens detected among 240 *M. haemolytica*. In addition, Abed et al. (2020) reported that three isolates only (60%) harbored ssa gene. The virulence of *M. haemolytica* is linked to different virulence genes

including lkt, especially lktC, gcp, and other genes, and characterization of these genes provides important information about the pathogenicity of *M. haemolytica* Singh et al. (2011) and Klima et al. (2014). The virulence genes detected in our study as shown in Fig. 4 & 5 and table 8 were similar to Klima et al. (2014) who detected lktC and gcp in all *M. haemolytica* isolates, El-Dokmak et al. (2015) who detected gcp gene in all tested isolates of *M. haemolytica* and Abed et al. (2020) who reported that Four of the tested *M. haemolytica* isolates 80% harbored both gcp and lktC (4 from 5 isolates). In our study, we confirmed the diagnosis of isolated bacterial species by PCR using unique primer sets as PCR assay is considered a specific, rapid and accurate technique for detection of different bacterial pathogens associated with cattle pneumonia (Bell et al. 2014).

Conclusion: Bovine respiratory disease is a serious problem affecting calves. *M. haemolytica*, *E. coli*, *S. aureus* and *Pseudomonas spp*. was isolated from pneumonic calves and confirmed by PCR. Virulence and antibiotic resistance genes for some identified bacteria were discussed. The presence of these pathogens threatens the livestock industry and is considered a public health concern for farmers and workers. In vitro susceptibility testing for the isolated bacteria revealed the presence of multidrug-resistant strains, suggesting the need for ongoing antimicrobial susceptibility monitoring. The phenotypic and genotypic characterization of the isolated bacteria is the most accurate diagnostic tool for an efficient treatment of BRD.

Author's contribution: HMA, OAA and EM design the study; HMA, OAA and EM methodology; HMA and EM data analysis; HMA and EM writing, reviewing and editing. All authors have agreed to the published version of the manuscript. All authors read and approved the final manuscript.

Table (2): Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
P. multocida Kmt1	ATCCGCTATTTACCCAGTGG GCTGTAAACGAACTCGCCAC	460	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Oie (2012)
M. haemolytica ssa	TTCACATCTTCATCCTC TTTTCATCCTCTTCGTC	325	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	Hawari *et al.* (2008)
M. haemolytica gcp	CGCCCCTTTTGGTTTTCTAA GTAAATGCCCTTCCATATGG	420	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	Klima *et al.* (2014)
M. haemolytica lktC	GGAAACATTACTTGGCTATGG TGTTGCCAGCTCTTCTTGATA	440	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	
M. haemolytica tetH	ATACTGCTGATCACCGT TCCCAATAAGCGACGCT	1076	94˚C 5 min.	94˚C 30 sec.	60˚C 40 sec.	72˚C 1 min	72˚C 10 min.	
M. haemolytica blaROB1	AATAACCCTTGCCCCAATTC TCGCTTATCAGGTGTGCTTG	685	94˚C 5 min.	94˚C 30 sec.	60˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	
M. haemolytica aphA1	TTATGCCTCTTCCGACCATC GAGAAAACTCACCGAGGCAG	489	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	
E. coli phoA	CGATTCTGGAAATGGCAAAAG CGTGATCAGCGGTGACTATGAC	720	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	HU *et al.* (2011)
Pseudomonas16S rDNA	GACGGGTGAGTAATGCCTA CACTGGTGTTCCTTCCTATA	618	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Spilker *et al.* (2004)

Table (3): The number of positive samples from examined calves:

Animal	Type of samples	No. of samples	Positive	Negative
Diseased calves	**Nasal swabs**	**60**	**30**	**30**
	Lung tissus	**40**	**25**	**15**
Apparently healthy calves	**Nasal Swabs**	**20**	**9**	**11**

Table (4): The prevalence of different types of bacterial pathogens among the examined samples:

Isolated microorganism	Source of samples			Total isolates no.
	Pneumonic calves N=100		Apparently health calves N=20	
	Nasal Swabs	Lung Tissue	Nasal swabs	
M. heamolytica	2	3	0	5(4.16%)
pseudomonas spp.	5	3	0	8(6.6%)
S. aureus	9	8	4	21(17.5%)
E.coli	14	11	5	30(25%)

Table (5): The frequency of the mixed bacterial isolates in the examined samples

Type of bacteria	Number
M. haemolytica +S. aureus+E.coli	2
M. haemolytica+ E.coli	1
M. haemolytica + S. aureus	1
pseudomonas spp.+ S. aureus	4
S. aureus+E.coli	10

Table (6): Serogrouping of *E. coli* isolates

E. coli Serogroup N=5	Number
O_{143}	2
O_{119}	1
O_{63}	1
O_{128}	1

Table (7): Antibiogram of the isolated different types of Bacteria

Antimicrobial	Disc. Conc. (µ)	*M. haemolytica* N=5		*E.coli* *N=10*		*pseudomonas spp* *N=8*		*S. aureus* *N=10*	
		R.	S.	R.	S.	R.	S.	R.	S.
Levofloxacin	5	-	5	-	10	1	7	1	9
Enrofloxacin	5	1	4	1	9	2	6	2	8
Gentamycine	5	3	2	3	7	3	5	4	6
Florphenicol	30	1	4	3	7	4	4	6	4
Cefquinome	30	-	5	6	4	2	6	2	8
Oxyteracycline	30	5	-	9	1	7	1	9	1
Sulphatrimethoprim	25	5	-	8	2	6	2	5	5
Amikacine	30	4	1	5	5	2	6	8	2
Kanamycine	30	4	1	8	2	5	3	8	2
Amoxacillin	25	5	-	8	2	5	3	6	4
Cefotaxim	30	2	3	7	3	4	4	7	3
Pencillin G	10	5	-	10	-	6	2	9	1

Table (8): Molecular characterization of *M. haemolytica* isolates , virulence and resistance genes

Sample No.	M. haemolutica ssa	lktC	Gcp	tetH	BlaROB1	AphA1
1	+	-	+	+	+	+
2	+	+	+	+	+	+
3	-	-	-	-	-	-
4	-	-	-	-	-	-
5	+	-	+	+	+	+
6	+	+	+	+	+	+
7	+	+	+	+	+	+

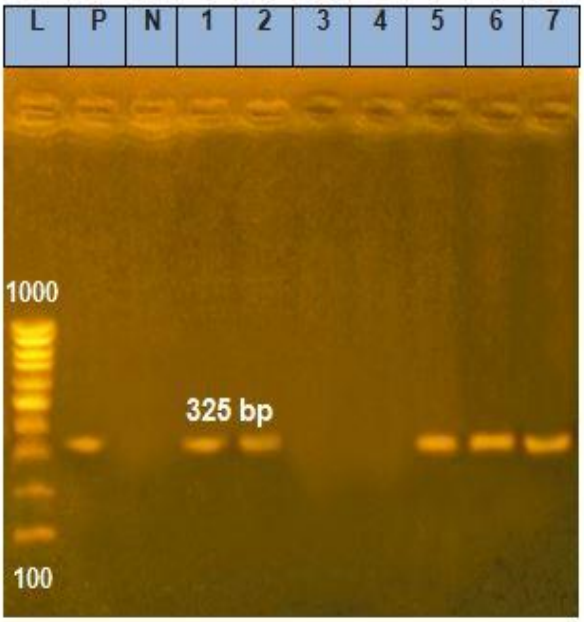

Fig. 1 Agarose gel electrophoresis of PCR products showing amplification of *M. haemolytica* (*ssa) gene* products at 325 bp . MWM-molecular weight marker (100 – 1000 bp DNA ladder), + control (Positive, Negative). Lanes (1.2.5.6.7) were confirmed as *M.*

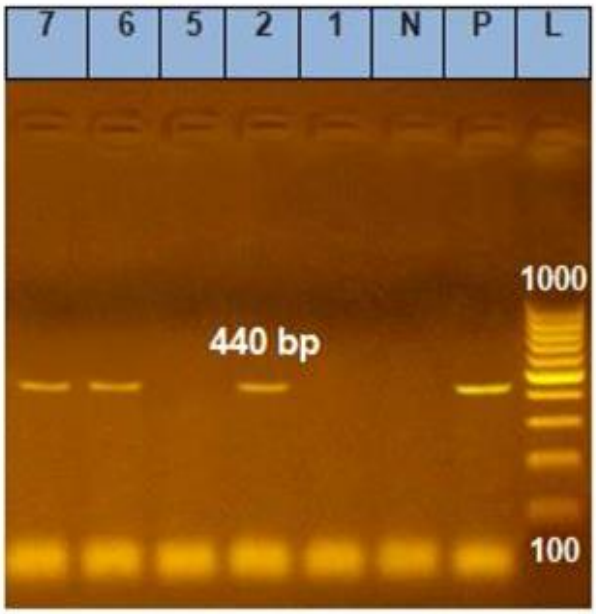

Fig. 2 Agarose gel electrophoresis of PCR products showing amplification of **M. haemolytica (lktC*) gene products** at 440 bp. MWM-molecular weight marker (100 – 1000 bp DNA ladder), + control (Positive, Negative) .

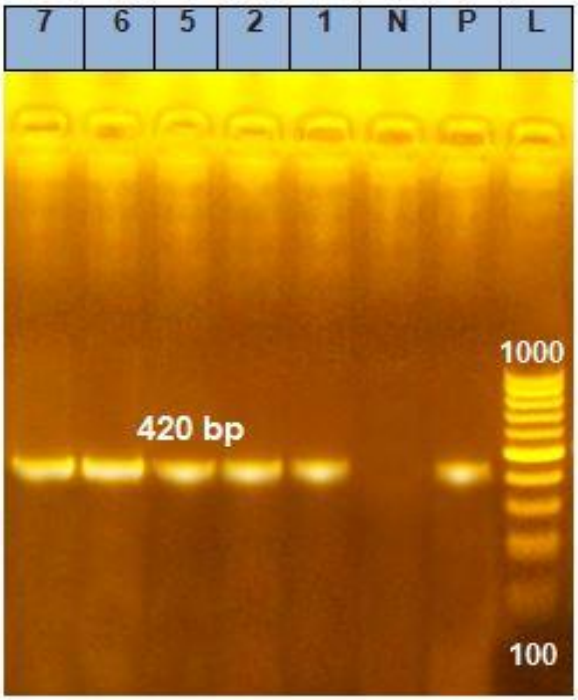

Fig. 3 Agarose gel electrophoresis of PCR products showing amplification of *M. haemolytica* (gcp*) gene* products at 420 bp . MWM-molecular weight marker (100 – 1000 bp DNA ladder), + control (Positive, Negative). All isolates were positive for this gene.

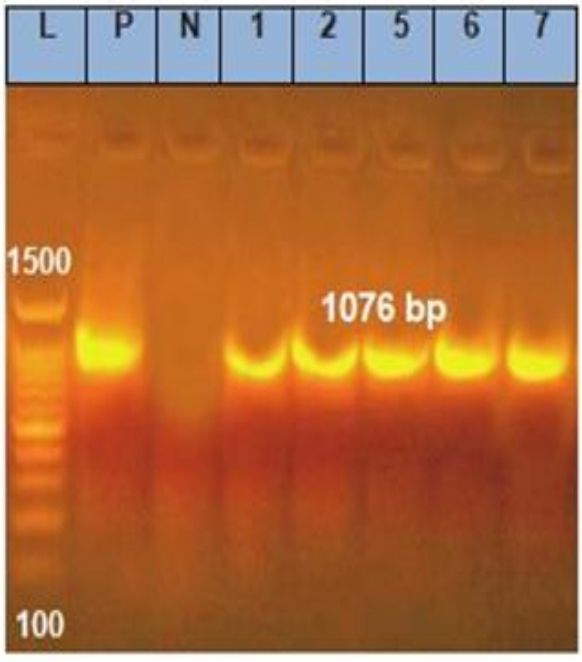

Fig. 4 Agarose gel electrophoresis of PCR products showing amplification of *M. haemolytica* (tetH*) gene* products at 1076 bp . MWM-molecular weight marker (100 – 1500 bp DNA ladder), + control (Positive, Negative). All isolates were positive for this gene.

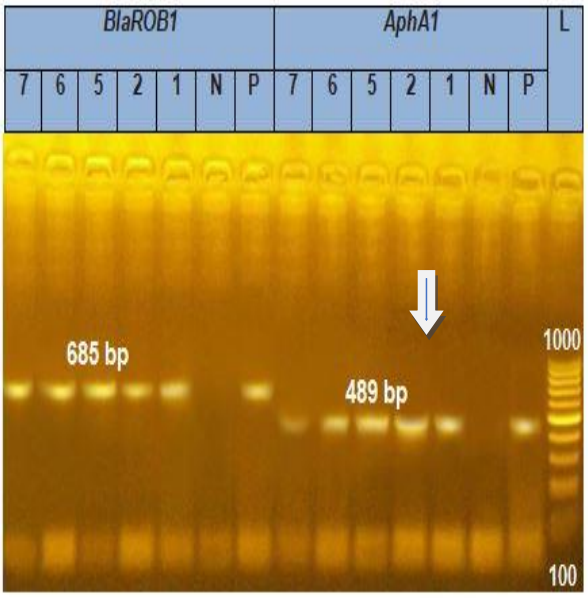

Fig. 5 Agarose gel electrophoresis of PCR products showing amplification of *M. haemolytica* (blaROB1*) gene* products at 685 bp , *M. haemolytica* (aphA1*) gene* products at 489 bp MWM-molecular weight marker (100 – 1000 bp DNA ladder), + control (Positive, Negative). All isolates were positive for two genes.

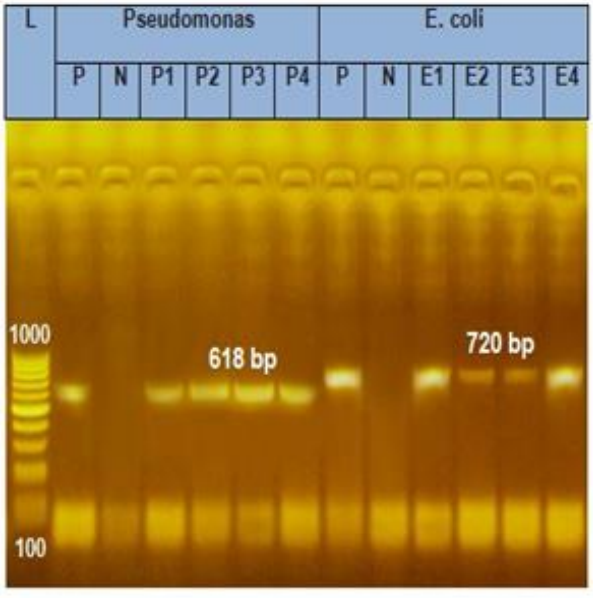

Fig. 6 Agarose gel electrophoresis of PCR products showing amplification of *Pseudomonas 16S rDNA gene* products at 618 bp , amplification of *E.coli phoA* gene products at 720 bp . MWM-molecular weight marker (100 – 1000 bp DNA ladder), + control (Positive, Negative) .

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