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Molecular and Phenotypical Characterization of *Mannheimia haemolytica* Isolated from Goats in Baghdad Province

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Abstract

Mannheimia haemolytica (M. haemolytica) is a gram negative bacterium which can infect humans and animals. It's commensal as a normal flora of the nasopharynx and tonsils in cattle, sheep and goats, pneumonic pasteurellosis is one of the most economically important infectious disease in goats worldwide prevalence. This study aimed to investigate the incidence of *M. haemolytica* by bacteriological and molecular characterization in goats. One hundred nasopharyngeal swabs were collected from apparently healthy field goats, seven lung tissue specimens and five nasal mucus swabs from slaughtered goats in Baghdad. All samples were cultured on Blood and MacConky agars. Biochemical tests and EPI20E kit were used for identification of the suspected colonies. 5 (4.46%) isolates of *M. haemolytica* were identified phenotypicaly and confirmed diagnosis by polymerase chain reaction (PCR) technique using two primers 16s rRNA and 12s rRNA genes .The results of this study concluded that identification of *M. haemolytica* by PCR was in accordance with those of phenotypic tests and it providing the basis for effective preventative strategies through epidemiological studies performance.

Keywords

Mannhimia haemolytica, Goats, Polymerase Chain Reaction (PCR), Baghdad

1. Introduction

Pneumonic pasteurellosis is one of the important respiratory diseases of cattle, sheep and goats worldwide distribution, It is usually caused by *Mannheimia haemolytica* biotype A [1]. It's considered a zoonotic disease, causes bronchiectasis, bronchitis, pneumonia, urinary tract infection, brain abscess, septicemia in

an infant [2] [3] [4] [5]. *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, previous or co-infection with certain respiratory viruses, mycoplasma or other types of bacteria [6]. Methods for its proper and rapid identification are critical to treating infected animals or gathering epidemiological data. Phenotypic tests have been described in details for identification of *M. haemolytica* and other species grouped within the genus *Mannheimia*, however, these test consist from 12 biochemical test and can be costly, time consuming and not specific for identification of *M. haemolytica* [7].

Few studies dealing with *M. haemolytica* infections were carried in Iraq, the bacteriological and serological methods were mostly performed for identification *M. haemolytica*. However, number of reports described prevalence of *M. haemolytica* in field animal by these methods only. The respiratory infection with *M. haemolytica* occurred in Iraq during the winters months, in (14%) and (4%) from infected and healthy sheep respectively [8]. In other reports, were reported *M. haemolytica* was isolated from sheep in Baghdad in (8.57%), whereas, *M. haemolytica* A serotype was isolate in (21.22%), while T serotype was isolated in (78.78%) [9]. Subsequently, the increase in the age of lambs accompanied by increase serotype T and decreases in serotype A [10]. As well as *M. haemolytica* isolated during an outbreak in mountain goat, deer and gazalles, the prevalence rate of the pathogen from nasopharyngeal swabs and blood samples was (68.0%) and (3.17%) respectively, while the prevalence rate of serotype A was (48%) and serotype T was (16%) [11].

Molecular methods of bacterial identification have proved beneficial to overcoming some limitations of the conventional biochemical and serological methods and improved sensitivity and rapidity [12] [13], Species-specific PCR assays are available for some bacteria within the family *Pasteurellaceae* [14], and the polymerase chain reaction was found useful, specific and time saving for identification of *M. haemolytica* using different oligonucleotide primers [15]. Improved *M. haemolytica* detection by PCR, two amplicons based PCR assay were used, targeted *M. haemolytica* specific mitochondrial 16S and 12S ribosomal RNA (rRNA) gene [16] [17]. The study aimed to identified *M. haemolytica* from apparently healthy & infected goats by phenotypic and molecular methods.

2. Material and Method

2.1. Samples and Bacterial Isolation

One hundred samples were collected from nasopharyngeal swabs of apparently healthy goats and twelve samples were taken from slaughtered goats (7 lung specimens and 5 nasal sinus mucus swabs).Then the samples were cultured in brain heart infusion broth (BHIB\Himedia, India) at 37˚C for 24 hr, and inoculated on blood agar (B.A\Himedia, India) with 5% sheep blood and MacConkey agar

(M.A\Himedia, India), then incubated under anaerobic condition at 37˚C for 24 h with 5% Co_2. Identification of the bacterial species was based on observation of their colonial morphology, Gram staining and biochemical characteristics (oxidase, catalase, indol, nitrate, urease, gelatin, simons citrate, motility, TSI, sugar fermentation tests) according to [18] [19]. The identification was confirmed by API20E kit. The API20E (Biomerieux Inc, USA) was done according to manufactures instructions.

2.2. Standard Strain of *M. haemolytica*

Standard strain of *M. haemolytica* (ATCC: P4WSO113) was supplied by Jovac Jordan Bio industries center. This strain was revived by adding 5 ml of normal saline to lyophilized bacteria, and inoculated in broth and media which were incubated for 48 hr at 37˚C with 5% CO_2.

2.3. Molecular Identification of *M. haemolytica*

2.3.1. DNA Extraction

Bacterial cells (up to 1×10^9) were cultured, in BHI broth, and overnight incubated, then were transferred to1.5 ml micro centrifuge tube then centrifuged at 16,000 g for 1 min, DNA was extracted using Presto Mini g DNA bacteria Kit according to manufacturer's instructions (Geneaid, KOBA). The extracted DNA was stored at −20˚C until use. The DNA concentration was measure by NANODROP-2000 spectrophotometer (Thermo Scientific Inc., USA).

2.3.2. Primers

Oligonucleotide primers for *M. haemolytica* were obtained from IDT (Integrated DNA Technologies/USA). The primer sequence of *M. haemolytica* 16SrDNA gene was (F-GCTAACTCCGTGCCAGCAG, R-CGTGGACTACCAG GGTATCTAATC) with size 304 bp [16] and the sequence of 12s rRNA gene was (F-TAACCCTTGTMCCTTTTGSATRRK, R-AGACTAACTTTTAAAGATACA GTGGG) with size 270 bp [17].

2.3.3. PCR Amplification Analysis

PCR amplification was performed on a final volume of 20 µl containing 10 Intron-Master Mix (KOBA) which contains (Taq plymerase, PCR buffer, Gel loading buffer and dNTPs), 2 µl (100 ng of DNA template) and 2 µl of 10 pmol for each primer. The amplification of gene was carried out with Master cycler (Eppendrof, Germany). Amplified products were separated by agarose gel electrophoresis (1% agaros containing 0.5 mg ethidium bromide in 0.5 × Tris-EDTA electrophoresis buffer) at 90 V/26 mA for 1 h and A 100 bp DNA ladder (Bioneer, Korea) was used as a molecular size standard, Gel documentation system.

3. Results

Results of one hundred nasopharyngeal swabs of apparently healthy field goats (0.00%) were negative for *M. haemolytica* (Table 1). While, 3/7 (42.85%) isolates

of *M. haemolytica* were isolated from the infected lungs tissue and 2/5 (40.0%) nasal sinus mucus isolates from slaughtered goats (Table 1).

Identification of *M. haemolytica* was done by study colonial morphology on B.A and M.A and showed minor differences between field isolates and standard strain. Lung isolates appeared on B.A as small, gray and rough colonies. While nasal isolates appeared as large, gray and mucoid (smooth) colonies (Figure 1). Lung and nasal isolates showed beta haemolysis on the B.A after 24 h. Lung isolates appeared as Pink pinpoint colonies on M.A, while nasal isolate appeared as mucoid pink colonies (Figure 2). While, standard strain of *M. haemolytica* appeared as small, gray and rough colonies on B.A haemolysis appeared after 48 h under neath the colony (Figure 1), and as dark pink pinpoint colonies on M.A (Figure 2). All isolates and standard strain were stained by Gram stain and methylene blue stain, the colonies appeared as G-, coccobacilli or short-rod singly or in pairs, clear bipolarity was appeared by methylene blue stain.

All isolates and standard strain examined by biochemical tests (Table 2). And EPI20E, minor differences were appeared between field isolates and standard strain.

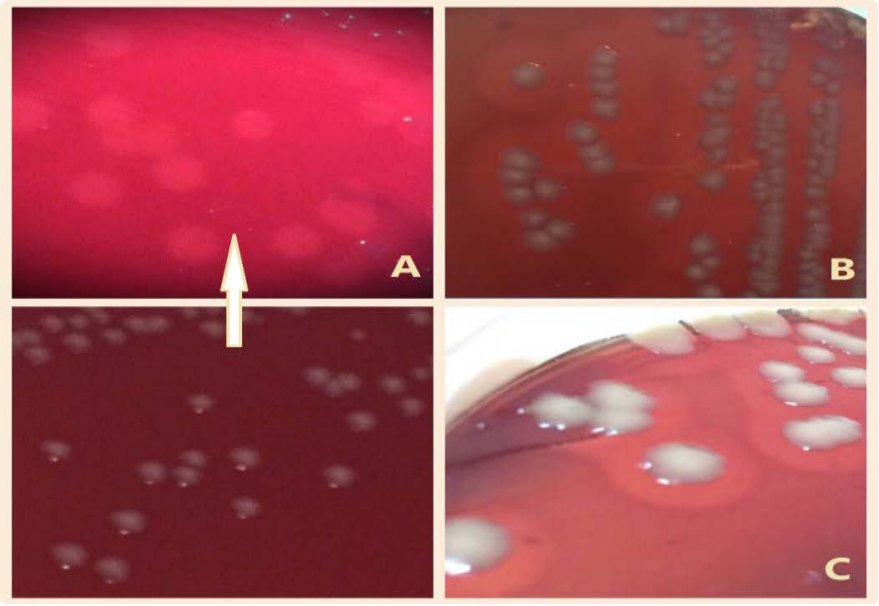

Figure 1. *M. haemolytica* colonies on B. A (A) Standard strain (B) and (C) Nasal& Lung isolates respectively.

Table 1. Source and isolation rate of *M haemolytica* from field and slaughtered goats.

Studies months	Type and sources of samples	No. of examined samples	No. of samples	Positive percentage %
October 2015 to February 2016	Field sample (nasopharyngeal swabs)	100	0	00.00%
March to April	Slaughter sample (lungs tissue)	7	3	42.85%
	Slaughter sample (nasal mucus)	5	2	40.00%
	Total	112	5	4.46%

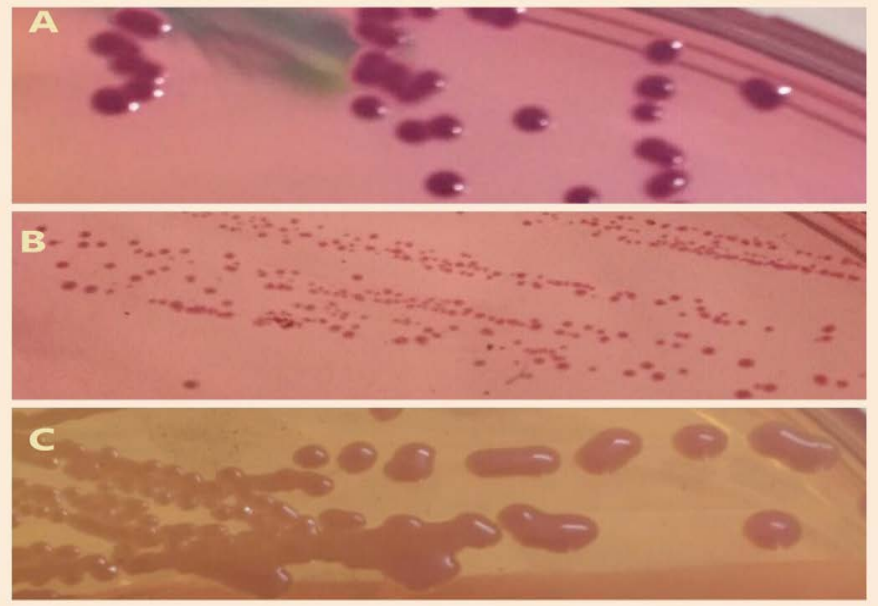

Figure 2. *M. haemolytica* colonies on M.A (A) Standard strain (B and C) Lung & Nasal isolates respectively.

Table 2. Comparison between recovered isolates and standard strain of *M. haemolytica* in Phenotypic test.

Phenotypic test	Recovered isolate	Standard strain
Colony and staining characteristic	On B.A: small, gray and rough or smooth colonies with beta haemolysis around colony. On M.A: lung isolate appeared as a pink pin point, while nasal isolate appeared as a pink mucoid Staining: G-, cocobacili, single or pairs and clear bipolar with methylen blue.	On B.A: small, gray and rough colonies with haemolysis appeared under Neath colonies. On M.A: growth colonies appeared dark pink like pin point. Staining : G–, cocobacili, single or pairs and clear bipolar with methylen blue
Indole test	+	+
Simmons citrate	–	–
SIM	–	–
TSI	yellow/yellow	yellow/yellow
Gelatinase test	–	–
Oxidase test	+	+
Catalase test	+	+
Urea test	–	–
Nitrate reduction test	+	+
Glucose acid	+	+
Lactose acid	(except nasal isolate–) +	+
Insitol acid	–	weak +
Sorbitol acid	–	+
Xylose acid	(except nasal isolate+)–	+
Rhamnose acid	+	+
Mannitol acid	–	+
Arabinose acid	–	+
Maltose acid	–	+

API20E results of the field isolates were appeared after 24 h, the results were (ONPG+, ADH−, LDC−, ODC−, CIT−, H2S−, URE−, TDA−, IND−, VP−, GLE−, GLU+, MAN+, INO−, SOR−, RHA−, SAC+, MEL−, AMY+, ARA−). API20 kit was specific for *Enterobacteriaceae* not for *Pasteurellaceae*, another tests were added in order to get best results (OX+), the digit code of *M. haemolytica* was (1004125). it was identified by using API web. While standard strain of *M. haemolytica* appeared after 48 hr. the results were (ONPG−, ADH−, LDC−, ODC−, CIT−, H2S−, URE−, TDA−, IND−, VP−, GLE−, GLU−, MAN+, INO+, SOR+,RHA−, SAC+, MEL−, AMY−, ARA−) and confirmative test (OX+), the digit code was (0000724)). It was identified as a *M. haemolytica* by using API web.

Confirm identification for all strains of *M. haemolytica* by PCR analysis, Assay was performed, all isolates were tested to present 16 s rDNA and 12 s rRNA genes. Hence, all strains were positive for 16 s and 12 s primers, they showed a specific 304 bp and 270 band respectively on agarose gel, no amplification was observed in control negative (**Figure 3** and **Figure 4**).

4. Discussion

Pneumonic pasteurellosis is one of the most economically important infectious diseases of goats, sheep and cattle with a wide prevalence throughout the continents [20] [21]. The negative results for isolation of *M. haemolytica* were observed from nasopharyngeal swabs during November 2015 to February 2016, while being isolated from slaughtered goats during March to April 2016. These results are in agreement with [22] who stated that calves may be negative for *M. haemolytica* on culture of nasal swabs. Also, pasteurellosis emerged as one of the major disease of animals adversely impacted by climate change [23]. Furthermore, they are in agreement with [24] who found that higher prevalence *M. haemolytica* was observed in spring and early summer. The negative results are

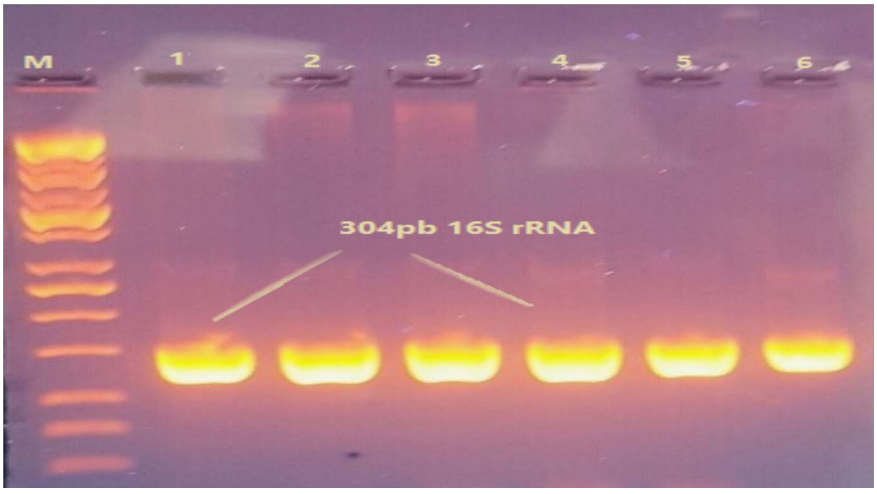

Figure 3. Agarose gel electrophoresis (1%) of amplified 16 srRNA gene (304 bp) of *M. haemolytica*, stained with ethidum bromide, Lane M: 100 bp ladder (1500 bp) Lanes 1 - 6: Five positive *M. haemolytica* isolates and the standard strain.

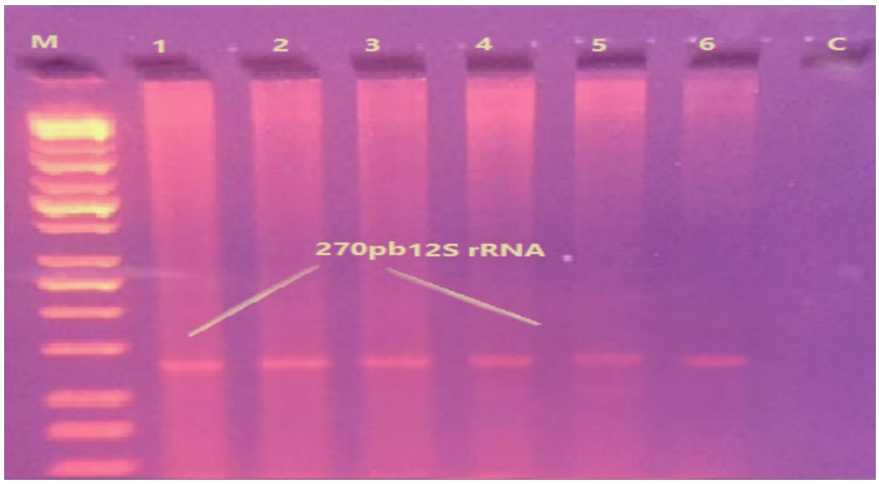

Figure 4. Agarose gel electrophoresis (1%) of amplified 12 srRNA gene (270 bp) of *M. haemolytica*, stained with ethidum bromide. Lane M: 100 bp ladder (1500 bp) Lanes 1 - 6: Five positive *M. haemolytica* isolates and standard, Lane c: negative control.

in agreement with [11] who pointed out for the negative isolation of *M. haemolytica* from nasopharyngeal swab of 3 healthy gazall, and with [25] who reported that *M. haemolytica* was not isolated from nasal swab of healthy animals, but 7(2.2%) isolates were obtained from diseased animal only. Also supported by [26] who reported that *M. haemolytica* is considered to be the major cause of bovine respiratory disease but it is rarely isolated from nasal swabs of healthy animals. The negative results for isolation of *M. haemolytica* from nasal swab are in disagreement with the isolation rate of *M. haemolytica* in the current study were 3 (42.85%) and 2 (40.00%) lung tissue specimens and nasal sinus mucus respectively from slaughtered goats. These results were higher and very convergent than 5/196 (2.55%) from *M. haemolytica* isolated from pneumonic lung samples from goats and sheep [27]. Also higher than [28] who reported that 5 (3.37%) isolates of *M. haemolytica* from goats and (15) isolate from cattle, buffalo and sheep from 148 samples of nasal swabs and lung tissue. Swabbing of the tonsils and nasal cavity of slaughtered sheep showed that *M. haemolytica* could be isolated from 95% of the tonsils and 64% of the nasopharyngeal swabs; the variation is likely to be caused by several factors including different isolation techniques, misidentification, and seasonal variation, [29]. The low isolation rate of *M. haemolytica* may be due delicate and very sensitive character of *Mannheimia* and these microorganisms not easily grow but need certain growth condition, these organisms flourished in a case in hydrolysate medium containing 15 individual amino acids, a mixtureof salts, vitamins, galactose and glucose [30]. The prevalence of *M. haemolytica* in previous investigation have shown a considerable variation, ranges between 8.9% and 96.2% of healthy sheep that carry these organisms in the nasal cavity [31]. The predisposing factors such as production stress in predominant climatic conditions of the region (hot weather, diurnal variations etc.) and changing weather patterns might have due to the stress established in the form of natural incidences of pneumonic pasteurellosis

in sheep [32].

For decades, microbiologists have sought improved pathogen identification through the use of phenotypic methods, but they still rely on phenotypic identification. Phenotypic tests were used in current study for identification of *M. haemolytica*. All characterization results of phenotypic tests for *M. haemolytica* (field isolates and standard strain) are in agreement with [18] who reported that the *M. haemolytica* appeared as a large or small, gray and rough or smooth colonies appeared on blood agar with haemolysis which appeared after 24 - 48 hr around or under neath the colonies, while it appeared as pink to red pinpoint colonies on MacConkey agar, except the nasal mucus isolate appeared as mucoid pink colonies on MacConkey agar, this result is closely agreement with [5] Also, the characterization of *M. haemolytica* isolates is in agreement with the findings reported by [33] that the isolate belong to *M. haemolytica* did not produce indole and grew on MacConkey agar.

All the 5 isolates and standard strain fermented glucose and rhamnose, the results are agreement with [34] Also, high variability for sugar fermentation was appeared between *M. haemolytica* isolates and standard strain, lactose fermentation observed by lung isolates and standard strain, of *M. haemolytica*, this result was similar to the finding reported by [35]. While, the nasal isolates were non lactose ferment, the results are in agreement with [5] While, the other carbohydrates fermentation of *M. haemolytica* were agreement with [5] [36] [37]. The variability observed in fermentation reactions of carbohydrates might be due to geographical variation of the isolates and use of chemotherapeutic agents as these factors will influence the enzyme profiles of microbes [38] or may be due to difference in the serotype of the isolates as serotype A ferments arabinose and serotype T ferments trehalose [18].

The five isolates and standard strain of *M. haemolytica* which positively identified by conventional biochemical's which were confirmed by API20E system. The results of API20E application for identification the field isolates were showed low discrimination for *M. haemolytica* with 18% .The results were agreement with [39] that proved the lower reliability of this system for the identification of *M. haemolytica*. Although API only claims to place *M. haemolytica* into the right genus, it was only able to correctly place 20% of the isolates in that category. The API system depending on specific recorded code number in service computer, hence, there are deviates strains of *M. haemolytica* have been different characteristics from standard strain and may be not reported in code number of service computer. While, API20E, application for identification the Standard stain of *M. haemolytica*, gave good result for identification of *M. haemolytica* with 92.7%, this result is in agreement with finding of [27] who successfully identified *M. haemolytica* by API20E. The five isolates and standard strain of *M. haemolytica* were positive for phenotypic and API20E tests, molecular identification was used to confirm identification.

All the 5 isolates and standard strain of *M. haemolytica* showed positive results for 16s rRNA gene and corresponds approximately size to 304bp, this result

is in agreement with finding of [16] were used the same primer which successively amplified to 304 bp and to sequencing as *M. haemolytica.* In addition, when 12s rRNA gene was used, all isolates also showed positive results and corresponds to anticipate size 270 bp. This result was in agreement with [17] who detected *M. haemolytica* directly from lung tissues and from bacterial culture by used 12s rRNA gene. The results of this study showed that PCR as a valuable tool for rapid detection of *M. haemolytica* in clinical samples from goats. In addition, it offers the opportunity to perform large scale epidemiological studies regarding the role of *M. haemolytica* in clinical cases of pneumonia and other disease manifestations in sheep and other ruminants, thereby providing the basis for effective preventive strategies.

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