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Original article

PREVALENCE OF *MANNHEIMIA HAEMOLYTICA* IN SYRIAN AWASSI SHEEP

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Summary

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The aim of the present study was to evaluate the prevalence of *Mannheimia haemolytica* in Syrian Awassi sheep. Between 2008 and 2012, 1520 nasal swabs from pneumonic and apparently healthy sheep, and 110 pneumonic lungs samples were collected and subjected to bacteriological, biochemical and PCR assays. A total of 191 isolates (11.7%) were identified as *M. haemolytica*, 44 (2.7%) were *M. ruminalis* and 18 (1.1%) were *M. glucosida*. All 191 isolates of *M. haemolytica* gave an amplified product of 1146 bp size by PCR when *lkta*A primer was applied. The highest rate of *M. haemolytica* isolation was from pneumonic lungs tissue (21.8%) and pneumonic sheep (14.1%), and the lowest was from apparently healthy sheep (8.5%). Geographical and seasonal distribution of *M. haemolytica* showed that the majority of the isolates originated from sheep reared in the Syrian Desert (30%) and Euphrates basin (26.7%), and a significant increase ($P \leq 0.05$) in the rate of positive isolates in summer and winter as compared to spring. These findings indicate that *M. haemolytica* may play an important role in development of pneumonia in Syrian Awassi sheep, especially in eastern parts of Syria, where drought and shortage of rain hit these zones periodically.

Key words: Awassi sheep, climatic factors, identification, *Mannheimia haemolytica*, PCR

INTRODUCTION

Mannheimia (Pasteurella) *haemolytica* is an opportunistic, Gram-negative, weak haemolytic bacterial pathogen that is one of the main causes of pneumonic pasteurellosis in sheep with reports of isolation from cases of mastitis and myocardium (Bisgaard, 1975; Mohamed & Abdelsalam, 2008). Strains of *M. haemolytica* are subject of constant revision, they formerly comprised 17 serotypes and 2 biotypes: biotype A (*M. haemolytica*) and T (*Pasteurella trehalosi*), symbolising the ability of the bacteria to ferment arabinose or trehalose, respectively (Gilmour & Gilmour, 1989). However, the recent advancements in molecular biology involving DNA hybridization studies and 16S rRNA sequencing proposed a reclassification of trehalose negative *M. haemolytica* complex to a new genus (*Mann-*

heimia) including 5 species (*M. glucosida, M. granulomatis, M. haemolytica, M. ruminalis*, and *M. varigena*) (Angen *et al.,* 1997, 1999a) On the other hand, *P. trehalosi* was moved to a new separate genus called *Bibersteinia trehalosi* (Blackall *et al.,* 2001). Therefore, it was suggested that extended phenotypic and genetic characterisation are necessary for proper identification of these organisms.

Awassi is the only sheep breed in Syria with large population of about 17 million heads (FAOSTAT, 2007) and despite its big role in animal industry, information on the prevalence of *M. haemolytica* is limited (Younan *et al.,* 1988; Younan & Wallmann, 1989). Diagnosis of sheep pasteurellosis in Syria is based on clinical signs, and serotyping, however, investigations on the specificity of serotyping as diagnostic tool demonstrated that serotyping does not represent a reliable method for identification because approximately 10% of isolates from ruminants are untypable (Younan & Wallmann, 1989). To date, no PCR assay has been used for detection of *Mannheimia* pathogens in Syria.

Due to the above-mentioned changes in taxonomy and the impact it may have on the understanding of the etiology, pathogenesis and epidemiology of these organisms, the present investigation aimed to evaluate the prevalence of *M. haemolytica* in Awassi sheep from Syria by identification and characterisation of the bacteria isolated from Awassi sheep and their lung using biochemical analysis and PCR technique and to estimate the influence of climatic factors on the rate of bacterial isolation.

MATERIALS AND METHODS

Samples collection

Samples collection was performed between 2008–2012 from Awassi sheep (aged between 3 months to 5 years) reared in Euphrates Basin (North east), Syrian

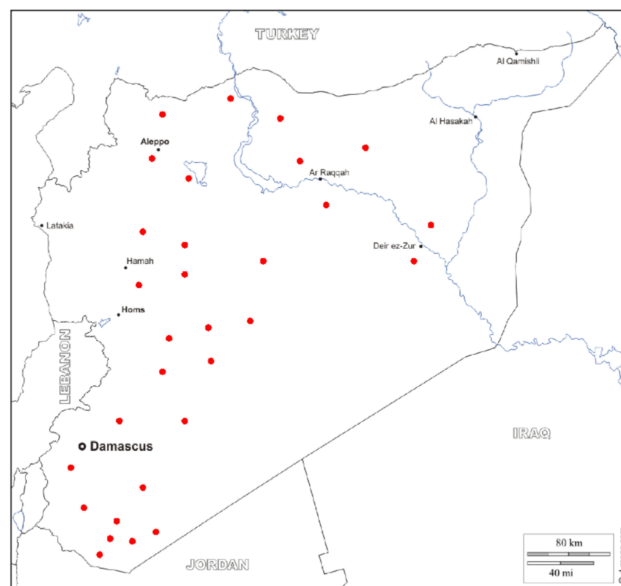

Fig. 1. General map of Syria showing selected sampling sites in the present study.

Table 1. Phenotypic characteristics of identified ovine isolates of *Mannheimia* species in the present study based on report of Angen *et al.* (1999b)

	M. haemolytica	*M. glucosida*	*M. ruminalis*
Gram-staining	–	–	–
Haemolysis on ovine blood	+	+	–
Motility	–	–	–
Oxidase	+	+	D
H&L, glucose	F	F	F
Urease	–	–	–
Indole	–	–	–
D (+) Mannose	–	–	–
Trehalose	–	–	–
L(+) arabinose	–	D	–
A-fucosidase	+	+	–
B-glucosidase	–	+	–
CAMP reaction	+ (week)	+	–

(+): ≥ 90 % strains are positive, (–) : < 10 % strains are positive. D: <90 and ≥ 10 positive. F: Fermentative.

Desert (East) and central zones (semi-arid steppe zones) of Syria (Fig. 1).

A total of 1520 nasal swab samples from 125 Awassi sheep herds (634 from pneumonic cases and 886 from apparently healthy sheep) were collected. In addition, 110 samples were collected from tissue of pneumonic lungs from slaughterhouses in Hama and Damascus prefectures (Central). The samples were placed in sterile test tubes containing 2 mL of transport medium (Amies medium), kept in ice and were then cultured in Erlenmeyer flasks containing brain heart infusion broth (BHI) (Difco) at 37 ºC overnight for further analysis.

Bacterial isolation and identification

Detection and identification of *M. haemolytica* was achieved by phenotypical and biochemical characters that were reported by Angen *et al.* (1999b) (Table 1) and the cultural, morphological and biochemical tests were carried out according to standard procedures of Cappuccino and Sherman (2001). Grown bacteria were plated on BHI agar containing 5 % ovine blood (BHI/blood) and on MacConkey agar. The inoculated plates were incubated aerobically and anaerobically at 37 ºC for 24–72 h. The plates were examined for *Pasteurellaceae*-like colonies that were grayish or yellowish, and showing a narrow zone of β-haemolysis. A single haemolytic colony was subcultured and tested by Gram-staining, catalase and oxidase (Fluka). All catalase positive, cytochrome-oxidase positive, Gram negative isolates were subjected for further analysis including 3% KOH test. Motility was examined by inoculation in semi solid medium (Brain Heart infusion broth, Difco with 0.25 % agar) followed by incubation at 22 ºC / 24 h. Catabolism of glucose was examined in Hugh and Leifson's medium (Merck). Urease was tested in Christensen's urea medium (Merck). Indole production was examined by adding Kovac's reagent to a 48 h old Heart Infusion Broth culture. Production of acid from: L(+) arabinose, D(+) mannose, and trehalose was examined as reported previously (Bisgaard, 1975) while production of α-fucosidase (ONPF) and β-glucosidase

(NPG) were determined with Rosco diagnostic tablets according to the manufactures instructions. CAMP-test was performed according to Christie *et al.* (1944).

PCR analysis

The PCR amplification was carried out with primers targeting *Lkt*A gene encoding for enzyme leukotoxin of *Mannheimia* species (Fisher *et al.,* 1999) with the following sequence: forward (5`-TGTGGA TGCGTTTGAAGAAGG-3`) and reverse (5`-ACTTGCTTTGAGGTGAT CCG-3`). Genomic DNA was extracted and purified using DNA extraction kit (BIOTOOLS, Cat. No. 21.002). PCR mixtures were prepared using 10–20 ng of template DNA, 0.4 µM of each primer, 1U of *Taq* DNA polymerase (Fermentase), 0.2 mM each of dATP, dCTP, dGTP and dTTP (Promega), 0.2 mM $MgSO_4$, and 3% dimethyl sulfoxide (DMSO). Amplification was done in a Bio-Rad T gradient thermocycler under the following conditions: four denaturation steps at 95 ºC, followed by 30 amplification cycles (1 min at 95 ºC, 1 min at 55 ºC, and 1 min at 72 ºC) and an extra extension step of 9 min at 72 ºC. The reference strain *E. coli* NIHJ was used as a negative control and PCR products were separated on a 1% agarose gel to which ethidium bromide was added and photographed under UV light.

Statistical analysis

Seasonal effect on the rate of bacterial incidence was statistically analysed. The Differences between percentages were evaluated by means of test of proportions using the *z*-test.

RESULTS

Of 1520 nasal swabs and 110 pneumonic lungs samples examined a total of 191 (11.7%) isolates were identified as *M. haemolytica* (167 from nasal swabs and 24 from lung samples), 44 (2.7%) were *M. ruminalis* and 18 (1.1 %) were *M. glucosida* (Table 2).

Isolation of *M. ruminalis* and *M. glucosida* from examined lungs were negative. The results of PCR assay with *Lkt*A primers are shown on Fig. 2. All the 191 isolates of *M. haemolytica* were found to give an amplified product of 1146 bp size, whereas no bands were observed when the same conditions of PCR were applied on strain *E. coli* NIHJ.

Table 2 demonstrates the relationship between the clinical condition of sampled sheep or lungs and the rate of isolation. Thus, the highest rate of *M. haemolytica* isolation was from pneumonic lungs (21.8%) followed by sheep suffered from pneumonia (14.1%) and the lowest was from samples of apparently healthy animals (8.5%). Geographical distribution of

Table 2. Incidence of *Mannheimia* spp. in respiratory tract of apparently healthy and pneumonic sheep

Source	Symptoms	Number of samples	Number (percentage) of positive isolates for:		
			M. haemolytica	*M. ruminalis*	*M. glucosida*
Nasal Swab	Apparently healthy	886	76 (8.5)	29 (3.2)	10 (1.1)
	Pneumonic	634	91 (14.1)	15 (2.3)	8 (1.2)
Lungs	Pneumonic	110	24 (21.8)	–	–
Total		1630	191 (11.7)	44 (2.7)	18 (1.1)

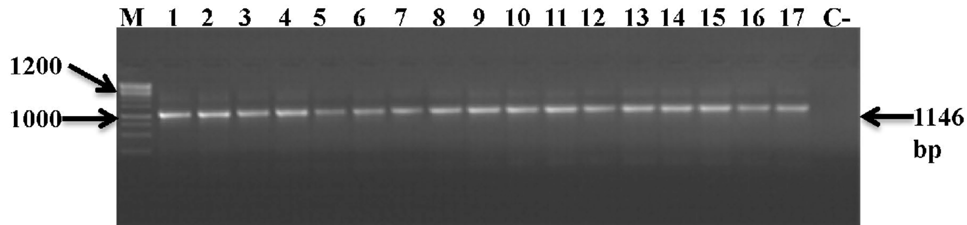

Fig. 2. PCR product of 17 field ovine isolates of *M. haemolytica:*M: DNA marker; lanes from 1 to 17: field isolates of *M. haemolytica* isolated from pneumonic lungs (lanes 1 to 7) and from nasal swabs samples (lanes 8 to 17); C-: reference strain *E. coli* NIHJ with *Lkt*A primer (negative control).

Table 3. Distribution of *M. haemolytica* isolates over the sampling zones of Syria

Zone		Number of samples	Number (percentage) of *M. haemolytica* positive
Euphrates basin		359	51 (14.2)
Syrian desert		294	58 (19.7)
Semi-arid	North	259	21 (8.10)
	Central	458	44 (9.6)
	South	260	17 (6.5)
Total		1630	191 (11.7)

Table 4. Seasonal variation in the incidence of ovine *M. haemolytica* isolation in Syria

Season	Number of samples[a]	No. of positive isolates / year				Total (%)
		2008	2009	2010	2011	
Spring	339	8	3	6	6	23 (6.78)
Summer	223	7	9	3	7	27 (12.11)*
Autumn	482	14	10	12	13	49 (10.17)
Winter	476	19	22	12	15	68 (14.29)*
Total	1520					167 (10.9)

[a] nasal swabs only; * P<0.05 compared to spring.

isolated *M. haemolytica* over sampled areas is shown in Table 3. The highest numbers of the isolates were from flocks living in the desert (58 or 30%), followed by Euphrates Basin (51 or 26.7 %) and central parts of Semi-arid step zone (44 or 23.13%), respectively.

The influence of climatic factors on the rate of *M. haemolytica* incidence is shown in Table 4. Significant increase (P≤0.05) in the rate of isolated bacteria has been observed in summer and winter as compared to spring (Table 4).

DISCUSSION

Of 1520 samples examined, a total of 191 (11.7%) isolates were identified as *M.*

haemolytica (Table 1). This study is the first extended phenotypic characterisation of a large collection of *M. haemolytica* from Syrian Awassi sheep. In previous studies, a limited number of samples from Awassi sheep in Syria were collected and serotyped (Younan *et al.,* 1988; Younan & Wallmann, 1989). The present study confirmed their findings. Gram staining of the nasal swabs and pneumonic lungs yielded Gram-negative bipolar coccobacilli, which is characteristic of *Mannheimia* species. All isolates presumed to belong to *M. haemolytica* were catalase and oxidase positive, did not produce indole, grew on MacConkey agar and trehalose negative (Table 1). These results are in agreement with the findings reported earlier (Angen *et al.,* 1999b; Tefera & Smola, 2002). Identification was further confirmed by PCR technique using *Lkt*A primer for identification of *M. haemolytica* (Fisher *et al.,* 1999). All *M. haemolytica* isolates that were positive by biochemical reactions were also positive by PCR. PCR assays have been described as a rapid method of identification of numerous bacterial species and thus were investigated in this paper as one of the tools to distinguish *M. haemolytica* from other bacterial species that are found in the upper respiratory tract of sheep. The leukotoxin (*Lkt*) operon was selected as a target because it is directly implicated in pathogenesis and deletion of *Lkt* genes of reduced virulence of *M. haemolytica* (Narayanan *et al.,* 2002).

In the present survey, the rate of bacterial isolation was influenced by two factors, the clinical condition of sampled sheep or lungs where, the highest rate of isolation was from pneumonic lungs and pneumonic sheep (Table 2) and the climatic variation of sampled areas where, significantly increased rate of isolated bacteria occurred in summer and winter compared to spring (Table 4). Previous investigations on the prevalence of *M. haemolytica* have shown considerable variation in the rate of bacterial isolation worldwide. Ranges between 8.9% and 96.2% of healthy and pneumonic sheep that carry these organisms in the nasal cavity have been reported (Biberstein, 1970; Al-Tarazi & Dagnall, 1997; Poulsen *et al.,* 2006). The variation is likely caused by several factors including clinical condition of sampled sheep, different isolation techniques, misidentification, and seasonal variation. In addition it was shown that climatic factors such as cold, wind, dust, shortage of rain and drought have significant impact on the development of pneumonic pasteurellosis in sheep and subsequently, on the rate of isolation (Lacasta *et al.,* 2008). About 57% of the isolates originated from Syrian Desert and Euphrates basin (Table 3). This might be explained by the influence of drought that affected those regions as reported by the United Nations Office of Disaster Risk Reduction (UNISDR). The last drought hit Syria was between 2007 and 2009 (Erian *et al.,* 2010). Its impact was clear in the eastern and north-eastern parts of the country where the population of Awassi sheep is high.

In conclusion, the results of the present study demonstrated that *M. haemolytica* is may be one of the most prevalent pathogenic agents that cause pneumonia in Syrian Awassi sheep. Successive droughts and shortage of rain may lead to enhancement the outbreak of enzootic pneumonia in Syrian Awassi sheep. Direct PCR testing of bacterial colonies from ovine samples significantly reduced the time required for identification of *M. haemolytica* from related ovine bacterial species. The importance of the findings

has to be investigated in further studies which may help in formulating better control measures against ovine pasteurellosis.

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