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Some Studies on Bacterial Causes of Respiratory Manifestations in Small Ruminants

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Abstract: This study was carried out on private flocks of sheep and goat in Dakahilia governorate, during December 2013 to February 2014, in which 140 head of sheep and goat were clinically examined, 35 sheep and 17 goats were suffering from a variety of respiratory manifestations. From each diseased sheep and goat, a double nasal swabs were taken for bacterial isolation and mycoplasma detection. Lung and liver samples were collected from 11 emergency slaughtered sheep and goats. Bacteriological examination of nasal swabs revealed isolation of *Escherichia-coli spp.* 12 isolates (23.07%), *Staphylococcus aureus* and Proteus vulgaris 5 isolates for each (9.61%), *Streptococcus pyogene* and *Klebsiella pneumoniea*. 4 isolates for each (7. 69%) and lastly *Haemophilus parainfluenzae* isolated from 3 nasal swabs. Bacteriological examination of lung and liver samples revealed isolation of *S. aeureus* from 6 samples and *S. pyogenes* from 7 samples, *E-coli* isolated from 5 samples and *H. parainfluenzae* from 2 samples. Mycoplasma gene (16srRNA) was detected in 20 nasal swabs out of 52 examined ones and not detected in lung samples using PCR technique. Histopathological examination revealed, fibrinous bronchopneumonia and neutrophilic infiltrations, necrosis of pulmonary tissue, severe inflammatory reactions with pulmonary emphysema which indicated predominance of bacterial infections and necrosis of hepatic tissue, whatever, it encountered of numerous caseated and purulent materials encircled by fibrous capsules reflected the high virulence of infectious agents. From this study, it can be concluded that bacterial causes of pneumonia in sheep and goats are numerous and should be differentiated, also mycoplasma has a main role in respiratory problems in small ruminants which could be detected directly from suspected samples using PCR.

Key words: Bacterial pneumonia · Mycoplasma · Sheep · Goat

INTRODUCTION

Respiratory disease is commonly encountered in sheep and goat flocks, affecting groups or individuals. It often involves a combination of infectious causes as well as predisposing managemental factors, potentially leading to significant losses [1].

Many species of pathogenic bacteria can cause bronchopneumonia. In acute outbreaks of pneumonia, *Mannheimia haemolytica* is the organism most frequently isolated from infected lung tissue[2]. A number of other bacteria, including *Pasteurella multocida, Mycoplasma, Escherichia coli, Actinomyces pyogenes, Streptococcus and Staphylococcus* were isolated from many infected lungs of sheep and goats [3]. *Haemophilus somnus* (recently reclassified as *Histophilus somni*) is associated with respiratory disease in American bison, domestic sheep and cattle [4].

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Mycoplasma pneumonia in sheep is usually caused by *Mycoplasma ovipneumoniae*, which results in an interstitial bronchopneumonia that is often subclinical. However, when clinical signs do occur they include a chronic, persistent, soft cough an ocular discharge and a mucopurulent nasal discharge, with signs potentially lasting for six months or more. Cases are usually afebrile, although acute cases can be febrile, anorexic and listless with respiratory distress; in many animals, more severe clinical signs are due to a secondary *M. haemolytica* infection. The most important Mycoplasma infections affecting sheep and goats are contagious caprine pleuropneumonia (CCPP) and contagious agalactiae syndrome (CAS) [5]. *Mycoplasma arginini* was involved as a causative agent of mild pulmonary changes in sheep and goats [6].

This study was carried out to determine the different causative agents of pneumonia and respiratory manifestations and its role in lamb morbidity and mortality, as well as to describe the pathological characters of bacterial pneumonia in small ruminants.

MATERIALS AND METHODS

Animals: This study was carried out at a period from December 2013 to February 2014 on a flock containing 140 head of sheep and goat with varying ages in Talkha city in Dakahilia Governorate. This flock was examined clinically for respiratory manifestations and the flock data were collected.

Samples

Nasal Swabs: Fifty-two nasal swabs from diseased sheep and goat (35 sheep and 17 goats) in duplicate were collected using sterile swabs and transport media. then send to the laboratory.

Tissue Samples: From 11 emergency slaughtered sheep and goat a total of 15 tissue samples were collected 7 sheep (7 lung and 2 liver samples) and 4 goats (4 lung and 2 liver samples) each sample was divided into two portions, one frozen at – 20°C for bacteriological examination and for mycoplasma detection and the other one preserved in 10% formalin for histopathological examination.

Bacteriological Examination: Bacteriological examination was performed on 15 tissue samples (11 lungs and 4 livers) and 52 nasal swabs. Samples were inoculated onto 7% sheep blood agar and MacConkey agar plates and were then aerobically incubated at 37°C for 24-48 h. Isolates were identified using standard microbiological techniques, colony morphology, staining and biochemical tests according to Quinn *et al.* [7].

Sero Grouping of *E. coli* Isolates: It was done at AHRI (serology unit) using pathogenic *E. coli* ‹O› antisera (MAST ASSURE).

Detection of Mycoplasma in Nasal Swabs Using Polymerase Chain Reaction (PCR)

DNA Extraction: Preparation of samples for DNA extraction was carried out according to Yleana *et al*. [8]. 5ml of 24-hour broth cultures of the samples were centrifuged for 10 minutes at 12000 rpm. The pellet was washed twice in 1 ml of phosphate buffered saline, pH 7.2 (PBS) and suspended in 50 µl PBS. The cell suspension was heated directly at 100°C for 10 min. in a heat block to break the cell membranes and then cooled on ice for 5 min. Finally, the cell suspension was centrifuged for 5 min. and the supernatant stored at - 20°C until use.

DNA Amplification: PCR amplification for Mycoplasma was performed in 50 µl reaction mixture consisting of 5 µl of 50 ng Mycoplasma genomic DNA, 25 µl of 2 x Master mix (Multiplex gen) VIVANTIS, 1 µl of 50 pmol of each primer, 0.5 mM MgCl2 and 35µl of DNase- RNase- free, deionized water. DNA amplification was performed as shown in Table (2). Following amplification, 5 µl of each amplicon was mixed with sample buffer and applied on agarose gel 1% (w/v) containing 0.5 µg of ethidium bromide. The samples were electrophoresed at 50 volts for 20 min on a horizontal electrophoresis unit. A 100 bp DNA ladder was used as molecular weight standard (VIVANTIS). After electrophoresis, the gel was visualized photographed.

Histopathological Examination: Tissue specimens (15) after careful postmortem examination were fixed in 10% formalin buffered solution and processed, 5-6 µm paraffin sections were stained with hematoxylin and eosin (H-E) according to Bancroft *et al.* [10], a sections were microscopically examined.

Table 1: Number of nasal swabs, lung and liver samples

		Slaughtered animals	
Samples Species	Nasal swab	Lung	Liver
Sheep	35	7	2
Goat	17	4	2
Total	52	11	4

Table 2: Primer and PCR assay of mycoplasma

Mycoplasma Species	Forwarded primer	Reverse primer	Product size (bp)	Thermal cycle	Reference
Sequence of 16S common gene for ruminant Mycoplasma	MunivF 5- AGA TC CTA CGG GAG CA GCA -3	MunivR 5' ACT GC GAT TCC GAC TTC ATG 3'	1000bp	Initial denaturation step at 94°C for 5 min., followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min. and extension at 72°C for 1.5 min. A final extension step at 72°C for 10 min.	Alberto *et al.* [9]

RESULTS

Clinical Examination: Clinical examination was carried out on a sheep and goat flock at Dakahilia Governorate, the flock consisted of (105 sheep and 35 goat) varying in their ages as shown in Table (3). The clinical examination of the animals for respiratory manifestations revealed that a total of 52 sheep and goat out of 140 at a percentage of (37.14%) showed signs of respiratory manifestations including nasal discharge, dyspnea, cough, rapid breathing as show in Fig. (1) & (2). Regarding to species, 35 out of 105 examined sheep at percentage of (33.33%) showed respiratory manifestation while 17 out of 35 examined goat at percentage of (48.57%) showed respiratory manifestations. The high percentage of respiratory manifestation observed at group > 2-years old in sheep (53.85%) and also in goat (65%) and there were11 emergency slaughtered sheep and goat at percentage of (21.15%).

Bacterial Isolation: The bacterial isolation and identification in Table (4) & Fig: (3) revealed that the highest isolation rate was *E.coli* spp.in which 12 isolates at percentage of (23.07%) followed by *S.aureus* and *P.vulgaris* in which 5 isolates for each at percentage of (9.61%). followed by *S.pyogenes.* and *K.pneumoniae.* by 4 isolates for each by (7.69%) and *H.parainfluenzae* isolated from 3 nasal swabs by (5.76%).

Bacteriological examination of lung and liver samples as shown in (Table 5) revealed that *S.aureus* was isolated from 6 samples, *S.pyogenes* was isolated from 7 samples, *E.coli* was isolated from 5 samples and *H. parainfluenzae* was isolated from 2 samples and it was observed that there is amixed infection, Serotyping of *E.coli* was illustrated in (Table 6).

Mycoplasma Detection: PCR technique was used to detect *Mycoplasma spp.* from nasal swab and lung and liver samples which revealed that mycoplasma were detected in 20 nasal swab out of 52 examined nasal swabs at percentage of (38.46%) While mycoplasma was not detected in tissue samples as explained in Table (7) and Fig. (4).

Histopathological Examination

Gross Examination: Lung showed mottled appearance of gray and red patches, crepitated sound in palpation, when serial sections were done, oozing of whitish gray pus was consistent in most cases after that *Staph Spp.* infection.

- The lung has soft consistency, dark red with green areas in color on its surface whitish to greenish pus was present when serial section after that *E -coli* infections.

Liver showing scattered whitish foci variable in diameter on its surface and deeply on its parenchyma (Figure 5 photo 4), on cut sections revealed creamy purulent exudate and friable cheesy materials.

Histopathological examination revealed fibrinopurulent bronchopneumonia where eosinophilic fibrinous exudate filling pulmonary alveoli and clusters of dead and living neutrophils, necrosis in pulmonary tissue

Fig. 1: Photos of respiratory manifestations in sheep and goats showing serous to mucopurulent nasal discharge.

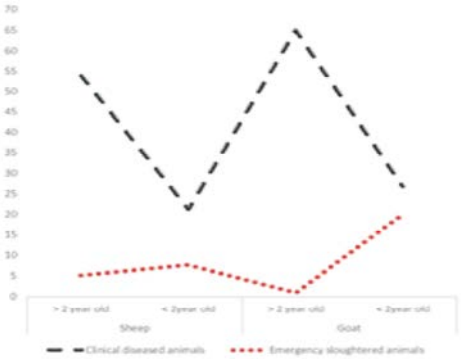

Fig. 2: Respiratory manifestations in sheep and goats in relation to their ages.(Needed to be reconstructed??

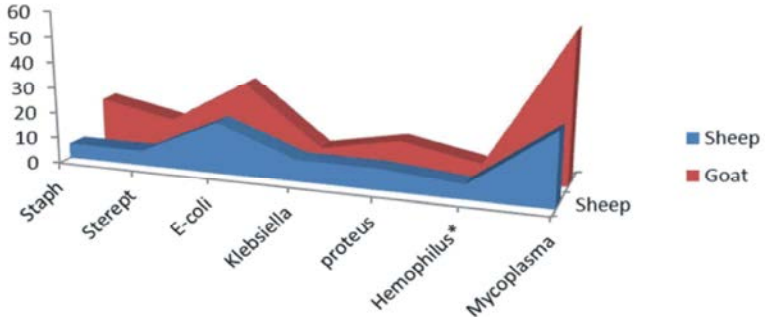

Fig. 3: Bacterial species isolated from nasal swab of sheep and goat with respiratory manifestations

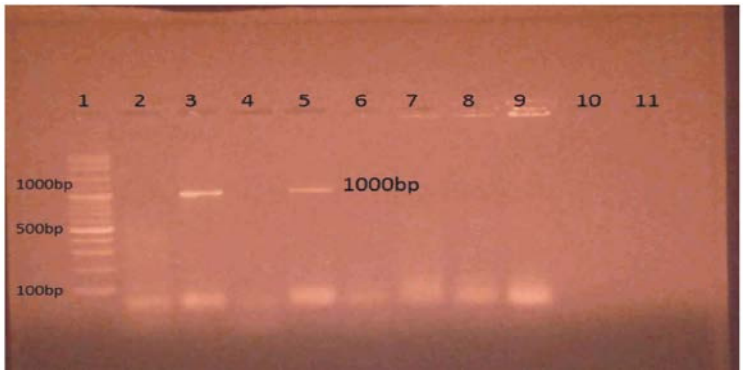

Fig. 4: Gel electrophoresis of PCR products of mycoplasma 16s rRNA gene. Lane 3and 5: The amplified products prepared from positive Nasal Swabs of diseased sheep, Lane 2: negative control, Lanes 4 &6-11 negative samples

Table 3: Results of clinical examination according to age.

Species	Age	Total examined	Diseased	%	Emergency slaughtered
Sheep	> 2 year old	39	21	53.85	2 (5.12%)
	< 2year old	66	14	21.21	5 (7.57%)
Total		105	35	33.33	7 (6.66%)
Goat	> 2 year old	20	13	65	1 (5%)
	< 2year old	15	4	26.66	3 (20%)
Total		35	17	48.57	4 (11.42%)
Total sheep and goat		140	52	37.14	11 (21.15%)

Table 4: Bacterial species isolated from nasal swabs of sheep and goat with respiratory manifestation

	Bacterial Isolation from Nasal Swab											
Species	*S. aureus*	%	*S. pygenes*	%	*E. coli**	%	*K.pneumoniae*	%	*P. vulgaris*	%	*H. parainfluenzae**	%
Sheep No = 35	2	5.71	2	5.71	7	20	3	8.57	3	8.57	2	5.71
Goat No = 17	3	17.64	2	11.76	5	29.41	1	5.88	2	11.76	1	5.88
Total No = 52	5	9.61	4	7.69	12	23.07	4	7.69	5	9.61	3	5.76

* Mixed infection between *H.parainfluenzae* and *E-coli* in 2 sample

Table 5: Bacterial species isolated from tissue samples obtained from emergency slaughtered sheep and goats with respiratory manifestations:

	Bacterial Species.			
Species	*S.aureus**	*S.pyogenes** #	*E. coli* #	*H.parainfluenzae**
Sheep No = 7 (7 lung & 2 liver)	3	4	3	2
Goat No = 4 (4 lung & 2 liver)	3	3	2	0
Total No. = 11	6	7	5	2

*Mixed infection between *S.aureus, H.parainfluenzae* and *E-coli* in 2 samples

Mixed infection between *S.pyogenes* and *E-coli* in 3 samples.

Table 6: Serotyping of E-coli isolated from nasal swabs and tissue of slaughtered animals

E. coli No = 17				
No	5	2	3	7
Serotype	O_{125}	O_{26}	O_{91}	O_{103}

Table 7: Detection of Mycoplasma from Nasal swabs and PM lesion from sheep and goat with respiratory manifestation using PCR

	Mycoplasma Detection					
	Nasal Swab			PM Lesion (lung, liver tissue samples)		
Species	No	Positive	%	No	Positive	%
Sheep	35	10	28.57	7	0	0
Goat	17	10	58.82	4	0	0
Total	52	20	38.46	11	0	0

and desquamation the epithelial lining of intrapulmonary bronchioles and presence of pulmonary emphysema were consistent with *Staph spp.* infections (Fig. 5 photo 1). In other cases, revealed extensive bronchopneumonia where fibrinous exudate obliterating pulmonary alveoli, interstitial congestion of pulmonary, necrosis in pulmonary tissue and neutrophilic infiltrating pulmonary tissue were consistent with E. coli infection (Fig. 5 photo 2). In other cases, necrosis in pulmonary tissue, congestion in interstitial blood capillaries and serofibrinous exudate filling pulmonary alveoli was consistent with *Haemophilus* spp. (Fig. 5 photo 3). Liquefactive to caseous necrosis of hepatic tissue, encircled with fibrous connective tissue capsules was present in most cases of Staph spp. infection. (Fig. 5 photo 4).

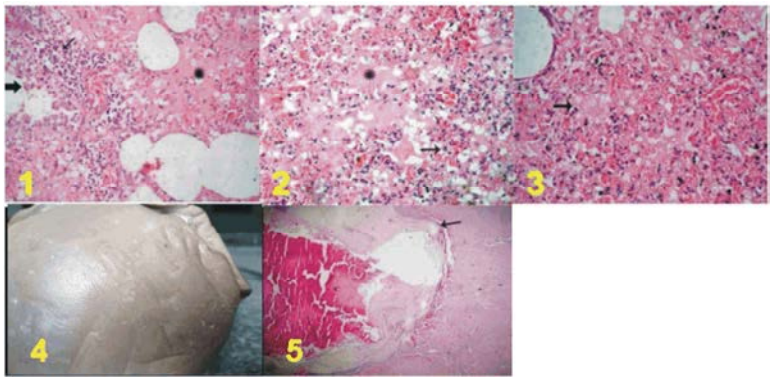

Fig. 5: Gross and histopathological examination
Photo 1: Lung showed bronchopneumonia where serofibrinous exudate filling pulmonary alveoli (rose shape) and neutrophilic infiltration (arrow) with severe necrosis of pulmonary tissue (thick arrow), *Staph. spp.* (HE, 400x)
Photo 2: Lung showing fibrinopurulent pneumonia where pulmonary alveoli filled with esinophilic fibrinous exudate obliterating it (rose shape) with neutrophilic recruitment into pulmonary tissue (arrow), E. coli. (HE, 400x)
Photo 3: Lung showing fibrinous pneumonia where pulmonary alveoli filled with esinophilic fibrinous exudate obliterating it (arrow) with severe congestion in inter alveolar capillaries in, Haemophilus spp. (HE, 400x)
Photo 4: Gross examination of liver showing multiple scattered whitish foci about 0.5 cm in diameter on its surface
Photo 5: Liver showing eosinophilic caseated material encircled by fibrous capsule (arrow). (HE, 100x)

DISCUSSION

In this study a total of 140 (105 sheep and 35 goat) were clinically examined, the clinical examination showed that 35 sheep and 17 goats were suffering from a variety of respiratory manifestations including nasal discharge (serous to mucopurullent), dyspnea, rapid breathing and cough and in some cases rise in body temperature, 11 cases were emergency slaughtered from respiratory manifestations during 3 days. According to species in sheep 35 out of 105 sheep (33.33%) showed respiratory manifestations while in goat 17 out of 35 examined (48.57%) showed respiratory manifestations. The high percent of respiratory manifestations observed in the group > 2-year-old age in sheep (53.85%) and also in goat (65%). These sings were mentioned by Bell [1], she mentioned that Clinical signs of pneumonia in sheep varied from mild cases which involve cough and oculonasal discharge, to more severe outbreaks some animals may be found dead and those that are affected and still alive often have a high temperature in the range of 40•4 to 42°C, hyperpnoea/dyspnea, loud respiratory sounds, serous nasal and ocular discharges and froth emanating from the mouth. The clinical course is usually 12 hours to death without treatment, but some animals can survive for as long as three days. Chronic cases can occur following initial recovery, resulting in persistent ill health and poor growth rates. Lambs up to 12 weeks of age will often have a septicemia with lung involvement. In outbreaks involving lambs, the morbidity can be up to 40 per cent, with mortality between 2 and 20 percent. Table (2) illustrated that the Bacterial species isolated from nasal swabs showed that *E.coli* spp was superior to other bacterial isolates as 12 isolates for *E-coli* by (23.07%) followed by *S.aureus.* and *P.vulgaris* in which 5 isolates for each by (9.61%) followed by *S.pyogenes.* and *K. pneumoniae*. by 4 isolates for each by (7.69%) then *H.parainfluenzae* isolated from 3 nasal swabs by (5.76%). While bacterial isolates from postmortem samples (lung, liver) were *S.aureus* isolated from 6 samples, *S.pyogenes* isolated from 7 samples, *E-coli* isolated from 5 samples and *H.parainfluenzae* isolated from 2 samples also there were mixed infection between bacteria.

The PCR technique is rapid, sensitive and efficient in mycoplasma diagnosis [11]. In our study we used PCR technique to detect the common 16srRNA gene of *Mycoplasma spp*. in nasal swab, the results in Table (3) showed that this gene was detected in 20 nasal swabs out of 52 nasal swabs examined by (38.46%), in sheep detected in 10 nasal swabs by 28.57% and in 10 nasal swabs in goat by (58.82%). The results go in the same way mentioned by Thiaucourt and Bolske [12] as they mentioned that Sheep are less susceptible to pulmonary mycoplasmosis than goats and also Chinedu *et al*. [13] who used Mycoplasma culture techniques followed by digitonin sensitivity testing, Species identification was

done using polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE). On Nasal swabs from 172 sheep and 336 goats from the Northeast, Northwest and South Senatorial Districts of Benue State, the Overall, Mycoplasma organisms were isolated from 131 (25.8 %) of the 508 SR examined. Prevalence rates of 18.1 and 29.8 % were recorded for sheep and goats, respectively. A total of 135 isolates of Mycoplasma belonging to three different species were identified: Mycoplasma ovipneumoniae (127), Mycoplasma arginini (7) and Mycoplasma mycoides subspecies capri (1). More than one Mycoplasma species were detected in four (3.1 %) of the 131 confirmed Mycoplasma positive cultures.

The histopathological examination revealed severe fibrinous and suppuartive bronchopneumonia, which correlated with bacterial isolation, also necrosis of pulmonary tissue and compensatory emphysema, due to damage and pulmonary obstruction in alveoli filled with exudates allowing the lesions to be progress in the pulmonary tissue and reflected clinically on animals with highly fatal manifestations. This is conducted with Ertan [14] which stated that most causes of mortalities in lambs are bacterial pneumonia which induces severe serious pathological alterations in the lungs. The presence of neutrophilic infiltration and moderate neutrophilic exudations and necrosis of pulmonary tissue in cases of *E. coli* infections is consistent with ORUÇ [15] who observed capillary hyperemia, edema and a few neutrophilic exudations were seen within the alveolar and bronchiolar lumina. Severe necrosis, aggregation of dead and living neutrophils in pulmonary tissue in *Staph spp. infected* cases was approved by Rashid *et al*. [16] . In liver the pathological lesions varied from focal necrosis to multifocal abscessations filled with caseated material and surrounded by fibrous capsule, indicated the escaping of causative bacteria and localization in the liver with subsequent serious pathologic effect on hepatic tissue.

CONCLUSIONS

We concluded that many bacterial spp. has been involved in pneumonia in small ruminants and has major economic importance, mycoplasma has a main role in respiratory problems in small ruminants, could be detected directly from samples using PCR. Isolated bacteria had many pathological changes in lung and liver tissues, which must be considered in treatment of respiratory manifestations in small ruminant.

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