Original Research Article

Antimicrobial and immunological studies on *Pasteurella multocida* and *Mannheimia haemolytica* recovered from calves affected with respiratory manifestations

El-Seedy, F.R.[a], Abed, A.H.[a, *]; Hassan, H.M.[b]; Nabih, A.M. [b]; Khalifa, E.[c] and Salem, S.E. [b]

[a] Bacteriology, Mycology and Immunology Department, Faculty of Veterinary Medicine, Beni-Suef University, Egypt.
[b] Animal Reproduction Research Institute, Giza, Egypt.
[c] Microbiology Department, Faculty of Veterinary Medicine, Matrouh University, Egypt.

ABSTRACT

Pneumonic pasteurellosis is the main cause of severe respiratory tract infections in calves and causing great economic losses. The objective of this research was to study the antimicrobial susceptibility of *P. multocida* and *M. haemolytica* and detection the effect of Lysozyme and Nitric oxide; as immune parameters, on most important bacteria causing pneumonic pasteurellosis in cattle calves. A total number of 406 deep nasal swabs and blood samples were collected from 406 bovine calves suffered from respiratory manifestations. Bacteriological examination revealed that the overall prevalence of both *P. multocida* and *M. haemolytica* was of 26.6%; 18.2% for *P. multocida* and 8.4%for *M. haemolytica*. *P. multocida* was singly isolated from 4.9% of cases. While it was mixed with *S. aureus*, *E. coli*, *Streptococcus* spp., both *S. aureus* and *E. coli*, both *S. aureus* and *Streptococcus* spp. and both *E. coli* and *Streptococcus* spp. with percentages of 4%, 1.2%, 2.2%, 1.7%, 3.2% and 1.0%, respectively. Meanwhile, *M. haemolytica* was isolated as a single isolate from 1.7% of cases while it was mixed with *S. aureus*, *Streptococcus* spp., both *S. aureus* and *Streptococcus* spp. and both *E. coli* and *Streptococcus* spp. with percentages of 2.7%, 1.2%, 2.5% and 0.2%, respectively. The in *in-vitro* sensitivity testing of all isolates showed high susceptibility to Fluoroquinolones and cephalosporins. On the other hand, high resistances were obtained against tetracyclines, penicillins and aminoglycosides. On the immunological level, the data of the existing research show that all respiratory affected calves record significant elevation of nitric oxide level in compare with normal control calves. However, all infected calves elucidate significant reduction of lysozyme activity.

[*] Corresponding author: Abed, A.H.; Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef 62511, Egypt. Tel. and Fax. (+2) 0822327982.
Email: aboabedelmasry@yahoo.com, ahmed.moawad@vet.bsu.edu.eg

1. Introduction

Bovine respiratory disease (BRD) is the main cause of severe respiratory tract infections in calves (Kirchhoff *et al*., 2014) and causing great economic losses due to reduction of average daily gain, feed efficiency, overall performance of beef calves and finally calves mortality (Härtel *et al.,* 2004 and Taylor *et al.,* 2010). It is a multi-etiological entity that is associated with several infectious agents (Fulton, 2009)

The disease manifests itself most often in calves within four weeks of weaning, when calves are sorted and sold to different farms. This gives it a common designate, "Shipping Fever"(Snowder *et al.*, 2006).

Pneumonic pasteurellosis refers to any of the disease conditions caused by bacteria of the genera Pasteurella or Mannheimia especially *P. multocida* and *M. haemolytica* are most commonly associated with pneumonia in cattle calves (Aly *et al*., 1990 and Asaye *et al.,* 2015). The disease manifests itself most often in calves within four weeks of weaning, when calves are sorted and often sold to different farms. This gives it a common nickname, "Shipping Fever" (Snowder *et al.*, 2006).

Infections with *P. multocida* and *M. haemolytica* are commonly managed by broad spectrum antimicrobials (Kehrenberg *et al*., 2001). Therefore, monitoring their antimicrobial susceptibility trends presents an important aid (Katsuda *et al.,* 2013).

Lysozyme is a small enzyme that attacks the protective cell walls of bacteria. Bacteria build a tough skin of carbohydrate chains, interlocked by short peptide strands, that braces their delicate membrane against the cells high osmotic pressure. Lysozyme breaks these carbohydrate chains, destroying the structural integrity of the cell wall. The bacteria burst under their own internal pressure (Magda, 2006). Lysozyme is a naturally found in body secretions such as tears, saliva, and milk. It functions as an antimicrobial agent by cleaving the peptidoglycan component of bacterial cell walls, which leads to cell death (Oliver and Wells, 2015).

Nitric oxide acts as intracellular signaling molecule or as neurotransmitter when produced in low quantities when is produced in high quantities for extended periods, it kills microorganisms and tumor cells. Nowadays, nitric oxide was identified as the effector molecule in killing a wide range intra and extra pathogens (Schmidt and Walter, 1994).

The objective of this study the antimicrobial susceptibility of *P. multocida* and *M. haemolytica* and detection the effect of Lysozyme and Nitric oxide; as immune parameters, on most important bacteria causing pneumonic pasteurellosis in cattle calves.

2. Materials and methods

2.1. Animal samples

A total number of 406 pneumonic bovine calves reared in different Governorates were examined during the period from January 2017 till December 2017. A total number of 406 deep nasal swabs collected under aseptic conditions for bacteriological examination from 406 calves affected with respiratory manifestation. Also, blood samples were collected from each calf for bacteriological and immunological investigations.

2.2. Bacteriological examination

Isolations of *P. multocida*, *M. haemolytica* and other bacteria were done according to Collee *et al.* (1996) and Quinn *et al.* (2002). The collected nasal swabs were inoculated under aseptic conditions into brain heart infusion broth (BHIB) and incubated aerobically at 37C for 6-8 hrs. A loopful from broth was cultured onto blood agar, MacConkey's agar and DAS media then incubated aerobically at 37C for 24 hrs. Cultivation of other bacteria were achieved using nutrient agar, MacConkey's agar, blood agar; eosin methylene blue media (EMB), mannitol salt agar; Baird Parker agar and modified Edward's media then incubated aerobically at 37C for 24-48 hrs. All the recovered isolates were identified morphologically using Gram's stain.

2.3. Blood smears

Two blood films were freshly prepared from each examined calf and stained with Leishman's stain for detection of Pasteurella bipolarity.

2.4. Biochemical identification of the bacterial isolates

All the recovered isolates were identified biochemically according to schemes described by Kreig and Holt (1984), Collee *et al.* (1996) and Quinn *et al* (2002). The suspected isolates of *P. multocida* and *M. haemolytica* were tested for haemolysis on blood agar and growth on MacConkey's agar as well as biochemical test; oxidase, catalase, indole, triple sugar iron agar medium, citrate utilization and sugar fermentation (glucose, lactose, sucrose and mannitol) tests.

2.5. Antimicrobial susceptibility testing

P. multocida and *M. haemolytica* isolates were tested for their antimicrobial susceptibility to 12 different antimicrobial discs. The antibiotics used were amoxicillin-calvulanic acid (30 µg), ampicillin-sulbactam (20 µg), cefiquinome (30 µg), cefotriaxone (30 µg), amikacin (30 µg), kanamycin (30 µg), sulphamethoxazol-trimethoprim (25 µg), levofloxacin (5 µg), ciprofloxacin (5 µg), oxytetracycline (30 µg), enrofloxacin (5 µg), and chloramphenicol (30 µg). Antimicrobial susceptibility testing was performed using disc diffusion method on Muller Hinton agar according to Clinical and Laboratory Standards Institute CLSI (2014). The antibiotic susceptibility was based on the induced inhibition zones according to the guidelines of the CLSI (2014).

2.6. Immunological studies:

2.6.1. Detection of lysozyme concentration

Assaying for lysozyme was done according to Schltz (1987), the clear zone ring diameters were measured to the nearest 0.1 mm with an enlarger-viewer (Kalesttad Laboratories, Inc., Austin, TX). For each lysoplate, the lysozyme concentrations in the samples were determined from a plotted standard curve against the corresponding clear zone ring diameter on linear axis.

2.6.2. Measurement of serum nitric oxide

The measurement of nitric oxide was assessed according to the assay described by to Rajaraman *et al.* (1998).

3. Results

3.1. Prevalence of *P. multocida* and *M. haemolytica* in calves

The overall prevalence of both *P. multocida* and *M. haemolytica* was 108/406 with a percentage of 26.6% arranged as 74/406 for *P. multocida* with a percentage of 18.2% and 34/406 for *M. haemolytica* with a percentage of 8.4% (Table 1).

Table 1. Prevalence of *P. multocida* and *M. haemolytica* isolates in calves.

No. of samples	*P. multocida*		*M. haemolytica*		Total	
	No.	%	No.	%	No.	%
406	74	18.2	34	8.4	108	26.6

%: Percentages were calculated according to the corresponding No. of samples.

3.2. Prevalence of single and mixed *P. multocida* and *M. haemolytica* isolation

P. multocida was singly isolated from 20 calves (4.9%) while it was mixed with other bacteria in 54 calves (13.3%). *P. multocida* was mixed with *S. aureus* (16 calves, 4%), *E. coli* (5 calves, 1.2%), Streptococci (9 calves, 2.2%), both *S. aureus* and *E. coli* (7 calves, 1.7%), both *S. aureus* and Streptococci (13 calves, 3.2%) and both *E. coli* and Streptococci (4 calves, 1.0%).

Meanwhile, *M. haemolytica* was singly isolated from 7 calves (1.7%) while it was mixed with other bacteria in 27 calves (6.7%). Totally, it was mixed with *S. aureus*, Streptococci, both *S. aureus* and Streptococci and both *E. coli* and Streptococci as follow: 11 calves (2.7%), 5 cases (1.2%), 10 cases (2.5%) and one case (0.2%), respectively. The mixed *M. haemolytica* isolates with *E. coli* or with both *E. coli* and *S. aureus* were not recorded (Table 2).

Table 2. Prevalence of single and mixed *P. multocida* and *M. haemolytica* isolation.

No. of samples		Isolation type	*P. multocida*		*M. haemolytica*	
			NO.	%	NO.	%
406		**Single isolation**	20	4.9	7	1.7
	Mixed bacteria	*S. aureus*	16	4.0	11	2.7
		E. coli	5	1.2	0	0
		Streptococci.	9	2.2	5	1.2
		S. aureus+ E. coli	7	1.7	0	0
		S. aureus+ **Streptococci**	13	3.2	10	2.5
		E. coli + **Streptococci**	4	1.0	1	0.2
		Total mixed	54	13.3	27	6.7
		Overall Total	74	18.2	34	8.4

%: Percentages were calculated according to the No. of samples.

3.3. Antimicrobial susceptibility testing

The *in-vitro* sensitivity testing of *P. multocida* isolates; either single or mixed infections showed high susceptibility to cefiquinome, levofloxacin and ciprofloxacin. On the other hand, they were highly resistant to oxytetracycline, ampicillin-sulbactam, amoxicillin-calvulanic acid and kanamycin (Table, 3).

The *in-vitro* sensitivity testing of *M. haemolytica* isolates; either single infection or mixed infections were highly sensitive to enrofloxacin, cefiquinome, levofloxacin and ciprofloxacin. On the contrary, they were highly resistant to oxytetracycline kanamycin, amikacin and amoxicillin-calvulanic acid (Table, 3)

Table 3. Antibacterial sensitivity testing of single and mixed *P. multocida* and *M. haemolytica* isolates.

Antimicrobial Discs	Discs Conc. (µg)	*P. multocida* isolates				*M. haemolytica* isolates			
		Single isolates		mixed isolates		Single isolates		mixed isolates	
		Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant
Amoxicillin-calvulanic acid	30	30%	70%	20%	80%	40%	60%	20%	80
Ampicillin-sulbactam	20	20%	80%	10%	90%	50%	50%	30%	70
Cefiquinome	30	90%	10%	80%	20%	80%	20%	80%	20
Cefotriaxone	30	60%	40%	30%	70%	70%	30%	50%	50
Amikacin	30	60%	40%	40%	60%	30%	70%	20%	80
Kanamycin	30	30%	70%	20%	80%	10%	90%	0.0%	100
Sulphamethoxazol-trimethoprim	25	70%	30%	50%	50%	40%	60%	40%	60
Levofloxacin	5	90%	10%	80%	20%	80%	20%	70%	30
Ciprofloxacin	5	90%	10%	70%	30%	70%	30%	70%	30
Oxytetracycline	30	10%	90%	0.0%	100%	0.0%	100%	0.0%	100
Enrofloxacin	5	70%	30%	60%	40%	90%	10%	70%	30
Chloramphenicol	30	50%	50%	30%	70%	40%	60%	30%	70

4.3. Results of immunological parameter:

Immune parameters related to single and mixed *P. multocida* isolates were recorded in table (4) show that all respiratory manifested calves record significant elevation of nitric oxide level in compare with normal control calves. Meanwhile, the infected calves with *P. multocida*+ Streptococci, *P. multocida*+ *S. aureus*+ *E. coli*, *P. multocida*+ *S.* *aureus*+ *Streptococci* and *P. multocida*+ *E. coli*+ Streptococci elucidates significant reduction of lysozymes. On the other hand, the infected calves with *P. multocida*, *P. multocida*+ *S. aureus* and *P. multocida*+ *E. coli* record no significant changes in serum lysozyme.

Table 4. Immune parameters related to single and mixed *P. multocida* isolates causing calves respiratory infection.

Bacterial isolates	Immune parameters	
	Nitric oxide	Lysozymes
P. multocida	9.29 ± 1.32	14.13 ± 0.95
P. multocida+ *S. aureus*	10.15 ± 0.82	12.66 ± 1.05
P. multocida+ *E. coli*	8.05 ± 0.91	12.15 ± 1.16
P. multocida+ Streptococci	11.33 ± 0.52	8.33 ± 0.13
P. multocida+ *S. aureus*+ *E. coli*	14.51 ± 1.03	7.39 ± 1.01
P. multocida+ *S. aureus*+ Streptococci	19.65 ± 0.93	7.61 ± 0.83
P. multocida+ *E. coli*+ Streptococci	14.33 ± 0.0	7.3 5± o.57
Normal	6.35 ± 0.29	11.66 ± 1.13

Immune parameters related to single and mixed *M. haemolytica* isolates were recorded in table (5) that show all respiratory manifested calves' record significant elevation of nitric oxide level in compare with normal control calves. However, all infected calves elucidate significant reduction of lysozyme activity.

Table 5. Immune parameters related to single and mixed *M. haemolytica* isolates causing calves respiratory infection.

Bacterial isolates	Immune parameters	
	Nitric oxide	Lysozymes
M. haemolytica	12.36 ± 1.39	7.05 ± 1.32
M. haemolytica+ *S. aureus*	14.62 ± 0.83	7.23 ± o.92
M. haemolytica+ Streptococci	14.66 ± 0.73	6.32 ± 0.88
M. haemolytica+ *S. aureus*+ Streptococci	15.46 ± 0.96	4.62 ± 0.39
M. haemolytica+ *E. coli*+ Streptococci	17.05 ± 0.72	5.02 ± 0.66
Normal	6.35 ± 0.29	11.66 ± 1.13

4. Discussion

One of the challenges of bovine respiratory medicine is early detection of clinical cases of BRD. BRD in calves is often referred to as a 'multifactorial disease' meaning that besides infectious agents, a multitude of environmental and management factors and their interactions are responsible for the outbreak of disease (Kabeta *et al*., 2015). Many of the infectious agents commonly involved in calf pneumonia are common inhabitants of the nasal passages of healthy animals. Many factors can weaken the host's immune system and/or damage the lining of the respiratory tract to such an extent that these pathogens are able to progress deeper into the respiratory tract and cause disease (Lopez, 2001). Any one or a combination of the environmental and management factors can make calves more susceptible to disease. Bacterial infections causing pneumonia in calves can possibly be fatal.

P. multocida and *M. haemolytica* are most commonly associated with pneumonia in cattle calves (Adamu and Ameh, 2007 and Asaye *et al.,* 2015). In current investigation, the prevalence of *P. multocida* and *M. haemolytica* were investigated and presented in table (1). The results revealed that the overall prevalence of both *P. multocida* and *M. haemolytica* was 26.6% arranged as 18.2% and 8.4% for *P. multocida* and *M. haemolytica*, respectively. These results were nearly similar to those reported by Zaki *et al*. (2002) who reported a high prevalence of *P. multocida* (19.9%) in comparison to *P. haemolytica* (8.8%) from 226 lung samples taken from pneumonic calves (1 day to 2 month old). Higher prevalences were recorded by El-Jakee *et al*. (2016) who recovered 88 *P. multocida* isolates from 256 calf nasopharyngeal swabs and lung tissues samples (34.4%). Also, Ahmed *et al*. (2015) estimated the prevalence of *Pasteurella* spp. in upper respiratory tract of cattle. The prevalence of *Pasteurella* spp. isolation was 38.88%. *P. multocida* prevalence was 30% while *M. haemolytica* prevalence was 20%.

In the same context Abera *et al*. (2014) found that the overall percentage of *P. multocida* (39.3%) and *M. haemolytica* (46.4%) , this results were higher than that mentioned by this study but this may possibly be due to the fact that The etiology of pneumonia is complex and multifactorial, thus the low rate of isolation of *P. multocida* and *M. haemolytica* from the examined animals in the current investigation may be due to other incriminated causes this results is supported by the results obtained by Garoia *et al*. (1982). On the other hand, lower prevalences were recorded by Lasisi *et al*. (2016) who investigated the prevalence of pneumonic pasteurellosis-caused by *P. multocida* and *M. haemolytica* in cattle and isolated *P. multocida* from six unhealthy lung tissue samples (7.22%) while *M. haemolytica* was isolated from one (1.22%) unhealthy lung tissue sample only.

Prevalences of single and mixed *P. multocida* and *M. haemolytica* infections were illustrated in table (2). *P. multocida* was singly isolated from 4.9% of cases. While it was mixed with other bacteria including *S. aureus*, *E. coli*, Streptococci, both *S. aureus* and *E. coli*, both *S. aureus* and Streptococci and both *E. coli* and Streptococci with percentages of 4%, 1.2%, 2.2%, 1.7%, 3.2% and 1.0%, respectively. Meanwhile, *M. haemolytica* singly isolated from 1.7% of cases while it was mixed with *S. aureus*, Streptococci, both *S. aureus* and Streptococci and both *E. coli* and Streptococci with percentages of 2.7%, 1.2%, 2.5% and 0.2%, respectively. The mixed *M. haemolytica* isolates with *E. coli* or with both *E. coli* and *S. aureus* were not recorded. The current findings may be due to presence of *S. aureus* and *Streptococcus* species as normal flora on the skin and oropharynx which may be flourished and causing diseases as a result of bad

hygienic measures or environmental and managemental stresses or any factors weakening the host's immune system and/or damage the lining of the respiratory tract to such an extent that these pathogens are able to progress deeper into the respiratory tract and cause disease (Kabeta *et al.*, 2015 an Lopez, 2001).

The study of Catry *et al*. (2002) indicated that the variety of compounds nowadays available to control bovine pasteurellosis is substantial. Examples are beta-lactam antibiotics such as aminopenicillins (+clavulanic acid) and extended spectrum cephalosporins (cefquinome, ceftiofur), fluorinated derivative of chloramphenicol. The results of *in-vitro* antimicrobial susceptibility tests of *P. multocida* and *M. haemolytica* isolates were illustrated Table (3). The in *in-vitro* sensitivity testing of *P. multocida* isolates showed high susceptibility to cefiquinome, levofloxacin and ciprofloxacin. On the other hand, high resistances were obtained against oxytetracycline, ampicillin-sulbactam, amoxicillin-calvulanic acid and kanamycin.

Meanwhile, the *in-vitro* antimicrobial susceptibility testing of *M. haemolytica* single isolates were highly sensitive to enrofloxacin, cefiquinome, levofloxacin, ciprofloxacin and ceftriaxone in a percentage of 90%, 80%, 80%, 70% and 70%, respectively. Meanwhile, high resistances were showed against oxytetracycline, kanamycin, amikacin and amoxicillin-calvulanic acid, in a percentage of 100%, 90%, 70% and 60%, respectively. Meanwhile in case of mixed infections, the isolates were highly sensitive to cefiquinome (80%) and 70% for each of levofloxacin, ciprofloxacin and enrofloxacin. On the other hand, complete resistances were shown against oxytetracycline and kanamycin (100% for each). Meanwhile amoxicillin/calvulanic acid and Amikacin were resistance in a rate of 80% for each.

Generally, the in *in-vitro* sensitivity testing of isolates showed high susceptibility to fluoroquinolones and cephalosporins. On the other hand, high resistances were obtained against tetracyclines, penicillins and aminoglycosides. The obtained data were agreed with the Katsuda *et al*. (2013) who found that the resistance of *P. multocida* to oxytetracycline was the most frequently observed phenotype among the isolates. Meanwhile, ceftiofur, cefquinome and enrofloxacin were effective antimicrobial agents, with no resistant isolates emerging over the course of the investigation. On the

contrary, these results disagreed with those reported by Abera *et al*. (2014) who detected the antimicrobial susceptibility of *P. multocida* and *M. haemolytica* isolates and found that the isolates were susceptible to most of the antibiotic discs used: amoxicillin, chloramphenicol, cephalexin, kanamycin and florfenicol. However, moderate resistance was observed to tetracycline, erythromycin and penicillin-G.Moreover, the current results somewhat agreed with Härtel *et al*. (2004) who found that the tested *Pasteurella* spp. showed no resistance to enrofloxacin or ciprofloxacin but differed in the susceptibility to other antimicrobials (ampicillin, penicillin, and tetracyclines). Also it agreed with Hendriksen *et al*. (2008) observed that all isolates of *P. multocida* were susceptible to ceftiofur, fluoroquinolones and differed in their susceptibility to ampicillin, amoxicillin+clavulanic acid, and tetracycline.

The obtained resistances against tetracyclines, penicillins and aminoglycosides might be attributed to the miss use of antimicrobial in animal treatment.

A nitric oxide has releasing solution (NORS) has been developed and shown to have potential in the prevention of bovine respiratory disease complex (BRDc) (Edwards, 2010). It was suggested that nitric oxide may protect against the development of BRDc by limiting deleterious inflammation while simultaneously increasing and enhancing the ability of the host to detect and respond to bacterial pathogens (Sheridan *et al*., 2016).

Lysozymes are naturally found in body secretions such as tears, saliva as well as milk and chicken egg white, but different sources lead to distinct antibacterial spectra, and specificity toward various types of peptidoglycans; Lysozyme is active mainly upon Gram-positive bacteria. Their antimicrobial function is due to cleavage of the peptidoglycan component of bacterial cell walls, which leads to cell death (Oliver and Wells, 2015). Lysozymesr N acetylhexosaminidases could lyse the cell wall of certain species of bacteria via hydrolysis of the β $(1\rightarrow4)$-glucosidic linkages of the peptidoglycan therein. Surprisingly, lysozymes show antibacterial effects even after irreversible inactivation (Ramos and Malcata, 2011).

In the current study, lysozyme and nitric oxide were estimated as immune parameters with detection their effect of on most important bacteria causing pneumonic pasteurellosis in cattle calves (Tables 4&5).

On the immunological level, the data of the existing research show that all respiratory affected calves record significant elevation of nitric oxide level in compare with normal control calves. However, all infected calves elucidate significant reduction of lysozyme activity. The present study was agreeing with the found by Civelek *et al*. (2007), they recoded that serum nitric oxide significantly rises in pneumonic calves in compare with control normal calves. Also, Tracey *et al*. (1995) and Lorente *et al*. (1997) found that plasma nitrite and nitrate concentrations significantly increased in an animal model characterized with endotoxaemia. Moreover, Hermeyer *et al*. (2012) investigated lung sections of the calves and indicated that the production of nitric oxide is potentially involved in the development of necrotizing lung lesions. Ackermann *et al*. (2012) found that serum lysozyme level suppressed in pneumonic animals. Moreover, several studies have examined lysozyme's potential as an exogenously administered biotherapeutic. The enzyme treatment also decreased lung tissue inflammation and decreased alveolar septal apoptosis (Teneback *et al*., 2013). Also, Seki *et al*. (2004) found that in chronic *P. aeruginosa* infected mice, secretion of lysozymes was significantly reduced by influenza virus infection in the whole lungs of chronic *P. aeruginosa* infected mice.

5. Conclusion

It was concluded that respiratory manifestations are a very important phenomena in bovine calves. *P. multocida* and *M. haemolytica* are the most common bacteria causing infection as the rate of infection with *P. multocida* and *M. haemolytica* were higher in bovine calves affections. The *in-vitro* sensitivity testing of isolates showed high susceptibility to fluoroquinolones and cephalosporins. On the other hand, high resistances were obtained against tetracyclines, penicillins and aminoglycosides. On the immunological level, the data of the existing research show that all respiratory affected calves record significant elevation of nitric oxide level in compare with normal control calves. However, all infected calves elucidate significant reduction of lysozyme activity.

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