Bacteriological and Virological Findings In Pneumonic Slaughtered Calves At Sharkia Governorate

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ABSTRACT

This study was carried out on 119 pieces pneumonic lungs and serum samples were collected from slaughtered calves at Sharkia Provinace Abbatoir for Bacteriological examination and Virological examination, the samples were cultivated on different media Pathogencity test , indirect haemoagglutincation test and agar gel diffusion test for bacteriological isolation. The number of isolation were105 (88.2%) isolates, the isolates represented by *Pasteurella multocida* B:6 (3.33%), *E.Coli* (20%)and *Staphylococcus aureus* (14.29%) but mixed isolates with *Pasteurella multocida* B:6 with *E.Coli* (11.43%). *Stahylococcus aureus* with *E.Coli* (10,48%) and *Staphylococcus aureus* with *E.Coli* with *Pasteurella multocida* B:6 (30.47%). The bacterial isolates were highly sensitive to *Enerofloxacine ,Gentamycine, Streptomycine and Resistantto Clindospectine*. In the virological investigation, bovine respiratory syncytial virus (BRSV) was isolated on MDBK cell lines and identified in 18 (15%) by using virus neutralization test. Antigens of BRSV was detected in 26 (21.8%) by using haemagglutination inhibition (HI) test. Moreover, antibodies against BRSV was detected in 43 (36.1%) by using ELISA in the collected serum samples.

INTRODUCATION

Respiratory diseases of cattle are represent a major problem of great economic importance due to the high threat leading to high death rates *(1)*. Pneumonia represent a major sign of affection to all ages of cattle especially calves *(2,3)*. Many infectious agents (as bacteria, viruses, parasites and fungi) play a role in lung infection. Climatic disorders, stress factors and environmental population are among the most predisposing factors to such infection. Many isolates of bacteria such as *Pasteurella* spp., *Streptococcus, Staphylococcus, Klebsiella* spp., *E coli, Mycoplasma* and circulating toxins are representing a major causes of pneumonia *(4.5)*.

Bovine respiratory syncytial virus (BRSV) is a member of genus *Pneumovirus* of the subfamily *Pneumovirinae* of the family *Paramyxoviridae* and was first isolated from cattle in 1970 *(6)*. The viral genome is a linear, single –stranded negative sence RNA *(7)*. The virus has an important role in the pathogenesis of pneumonia in cattle, especially in calves and yearlings *(8)*. In calves < 6 months induces acute interstitial pneumonia with alveolitis and bronchiolitis *(9)*.

Signs of BRSV infection in older cattle are mostly moderate or inapparaent *(10)*, but severe signs of respiratory tract infection have been observed in dairy cows *(11)*. BRSV infection is chrarcterized by sudden onset of fever, rhinitis, coughing, respiratory distress, increased bronchial sounds, abdominal breathing and reduced appitite *(12)*.

Pathological lesions in cattle with severe disease is characterized by bronchiolitis, multifocal and interstitial edema, emphysema and in some cases progressing to severe bronchopneumonia may ended with death *(13)*.

Diagnosis of BRSV infection by virus isolation is difficult to be carried and often requires blind passages of suspect material in cell culture before viral cytopathic effects (CPE) are evident. BRSV can be isolated on Madian-Darby bovine kidney (MDBK) cell lines, and show CPE 5-7 days post inoculation after 3-5 passages. The virus develops numerous syncytial cells and intracytoplasmic eosinoohilic inclusion bodies *(14,15)*.

Fluroscent antibody test is more rapid and simple over virus isolation in diagnosis of BRSV infection. Serological investigation such as SNT, CFT. IP and ELISA is considerably more fruitful in viral diagnosis *(16)*. BRSV infection can be accurately diagnosed by using reverse transcriptase polymerase chain reaction *(17)*.

Although many researches was done in BRSV in Egypt *(18,19)*, it still one of the greatest problems that threaten livestock and causes heavy losses among susceptible animals.

The aim of this study was to detect the main bacterial and viral causes of calves pneumonia in Sharkia Governorate and determine the sensitivity test of the isolated bacteria in order to obtain an available and specific treatment. Trails of isolation and identification of BRSV from the collected lung tissues, detection of BRSV antigen in the collected lung tissues as well as detection of antibodies in the collected serum samples were carried out.

MATERIAL AND METHODS

Material

1-Animals

119 male fatting steer calves were selected aged 2 to 5 years suffered from respiratory signs (coughing, mucopurelent nasal discharge). The affected animals were collected from different markets and transported to Belbis abbatoir, Sharkia Governorate.

2-Samples

A-lung tissues

A total of 119 samples of pneumonic lungs showing gross lesions of pneumonia (as swelling and congestion), were collected after slaughtering in sterile plastic bags on ice and divided into two portions and transported to the laboratory for bacteriological and virological examination during the period from December 2005 to April 2006.

B-serum samples

Blood samples were collected from jugular vein using a sterile needle for each animal in a sterile labeled venoject tube. All tubes were transported as early as possible on an ice packed thermos to the laboratory where it centrifuged at 2000 r.p.m for 10 minutes to obtain clear serum samples. The sera were separated in a sterile capped vials and inactivated at 56°C for 30 minutes to remove non specific inhibitors and preserved at -70°C tell used.

3-Media

Nutrient broth (Difco), Peptone water (Difco), Sugar media, MacConkey and 7% Sheep blood agar prepared *(20)*.

4-Stain

Gram stain ,Giemsa stain *(20)*

5-Laboratory Animals

15 mice (3weeks age) were obtained from Department of Pathology, Faculty of Veterinary Medicine–Zagazig University, used for purification of *pasteurella (P)* isolates.

6-Antibiotics

Different antibiotics were used Enerofloxacine (5ug) Excenel (30ug) ,Gentamycine (10ug), Streptomycine (10ug) ,Amoxycilline (10ug), Clindospectine (30ug)and pencillin (10iu) were obtained from Animal Health Dep. *Pfizer* co. and used for sensitivity test of the isolated bacteria.

7- Reference BRSV and BRSV antiserum

Reference BRSV and BRSV antiserum were originally obtained from National Veterinary Services Laboratory – Ames, Iowa, USA and were kindly supplied by Dep. Of Virology, Animal Health Research Institute – Dokki – Giza.

8- Cell culture

A continuous cell line of Madian Darby Bovine Kidney (MDBK) cells were supplied by Rinder Pest like Disease Department, Veterimnary Serum and Vaccine Research Institute- Abassia, Cairo. The cells was grown and propagated using modified Egle's minimum essential medium (EMEM). The MDBK cells were used for virus isolation and identification.

9- ELISA kits

ELISA kits for bovine respiratory syncytial virus antibody detection were supplied from Bio. X Diagnostics – Site du Complexe des Postes – 22, rue J. Wauters – 5580 Jemmelle – Belgiue, and was used for detection of antibodies against BRSV in the collected sera.

Methods

Bacteriological investigation

Each specimen was cultured into nutrient broth for 24 hours at 37C and then loopful was taken and sub-cultured onto each of the different following medias; nutrient agar, 7% Sheep blood agar and MacConkey,s agar. The inoculated plates were incubated at 37°C for 48 hours . Growing colonies were picked up and purified by subculture on nutrient broth and then cultured on selective media and the isolates were identified morphologically, Gram,s stained as well as their biochemical characters were studied *(20).*

On the other hand coccobacilli were inoculated in laboratory mice for pathogenicity *(21)*.The serological identification of *pasteurella (p)* organisms using *P.* antisera (Vet. Serum and Vaccine Research Institute, Abbasia, Cairo) were carried *(21)* for capsular typing and, a somatic typing were carried out *(22)*.

Sensitivity test; Susceptibility of the most predominant pathogenic isolates to different chemotherapeutic agents was tested by disc diffusion method *(23)*.

Virological investigation

1- Detection of BRSV in cell culture

The virus was isolated according to the previously described protocol *(24)*. Isolation attempts were done using 119 samples in trail to isolate BRSV on MDBK cells. MDBK cells were distributed in plastic 96 wells tissue culture plate for 70% confluence, the growth media was discarded and 50 µl from each sample was inoculated into triplicate wells. For each plate, cell control and virus control were included. The plates were incubated for one hour for adsorption, the virus inoculum and the plates was washed using 200µl of EMEM added to each well, then incubated at 37°C for 5-7 days with daily examination for recording the development of cytopathic effect. After 5-7 days the virus was harvested and used for subsequent passages. After the 3rd passage, the cells were harvested and kept at – 70 °C for virus identification.

2- Identification of isolated virus

Identification of the isolated virus was carried out by virus neutralization test *(25).*

3-Detection of antigens

Detection of viral antigens of BRSV was carried out by haemagglutination inhibition (HI) test. *(26)*

4- Detection of the antibodies

Detection of antibodies against BRSV in the collected serum samples by using ELISA test was carried out according to the described method by the producing company (Bio. X Diagnostics – Site du Complexe des Postes – 22, rue J. Wauters –5580 Jemmelle – Belgiue).

RESULTS

The biochemical identification of bacteria isolated were E. coli , Staphylococcus aureus and Pasteurella identified by pathogenicity test while serological identification using indirect haemoagglutincation test and agar gel precepatition test, showed that it the isolate was belongs to B:6

The results were tabulated in Tables 1, 2, 3, 4, 5 and 6.

Virus isolation

Virus isolation was applied by injection of prepared tissue suspension on MDBK tissue culture cell lines, and with daily observation of the injected tissue culture for 5-7 days.

The positive samples were showed characteristic cytopathoic effect (CPE) which firstly occur in the form of cells aggregation around the long axis, then fusion of the cells forming multinucleated "giant cells" end to shape of syncytia.

Table 1. Percentage of bacteria isolated from congested lungs of slaughtered calves.

Numberof slaughtered calves	Isolated bacteria							
	Number of positive cases		Number of negative		Single isolate		Mixed isolates	
	Number	%	number	%	number	%	number	%
119	105	88.24%	14	11.76%	50	47.62%	55	52.38%

Table 2. Types and number of bacteria isolated from pneumonic lungs.

No	Pure culture			Mixed culture		
	Bacteria	**No**	**%**	**Bacteria**	**No**	**%**
1	*E. coli*	21	20	*E. coli* with *Pasterulla mltucida B:6*	12	11.43
2	*Pasterulla mltucida B:6*	14	13.33	*Staphylococcus aureus* with *E. coli*	11	10.48
3	*Staphylococcus aureus*	15	14.29	*Staphylococcus aureus* with *E. coli* with *Pasterulla mltucida B:6*	32	30.47
Total		50	47.62		55	52.38

Table 3 . Sensitivity test on bacteria isolated

Antibiotic	*E.Coli* 30		*Pasteurella Multocida B:6* 33		*Staphylococcus aureus* 42	
	R	S	R	S	R	S
Enerofloxaccine 5ug	5 17%	25 83%	10 30%	23 70%	5 12%	37 88%
Excenel 30ug	7 23%	23 77%	11 33%	22 67%	17 40%	25 60%
Gentamycine 10ug	9 30%	21 70%	6 16%	27 84%	12 28%	30 72%
Streptomycine 10ug	8 26%	22 74%	3 9%	30 91%	11 26%	31 74%
Amoxycilline 10ug	25 83%	5 17%	32 97%	1 3%	6 14%	36 86%
Clindospectine 30ug	28 93%	2 7%	15 45%	18 55%	19 42%	23 58%
Pencillin 10iu	30 100%	0 0%	33 100%	0 0%	10 24%	32 76%

R= Resistance S= Sensitive %= Percentage

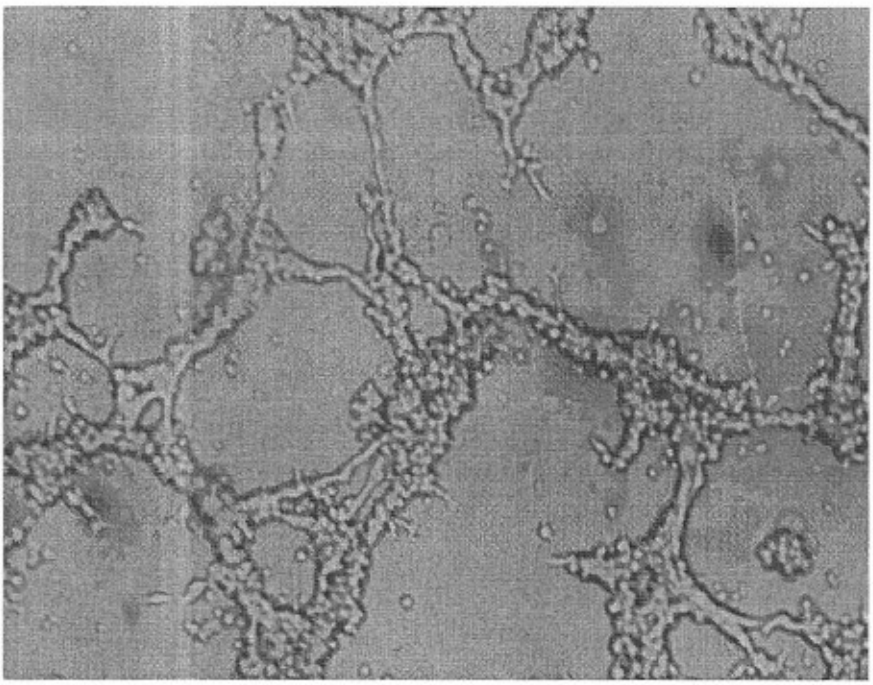

Fig. 1. MDBK cells after 7 days from inoculation (shape of syncytia)

The results of virus neutralization revealed that 18 samples (15%) were positive the presence of bovine respiratory syncytial virus (BRSV).

Detection of BRSV antigen in the prepared lung samples using haenagglutination inhibition test revealed that 26 samples (21.8%) were positive for the presence of BRSV antigen in the collected lung tissues.

Detection of BRSV antibodies in the collected serum samples from the examined animals using ELISA test revealed that 43 samples (36.1%) were positive for the presence of antibodies against BRSV.

Table 4. Results of serum neutralization test for identification of bovine respiratory syncytial virus. .

Age of animals	Collected samples	Positive samples	%
2 – 3 years	43	7	16.2
3 – 4 years	55	6	11
4 – 5 years	21	5	23.8
Total	119	18	15

Table 5. Results of haemagglutination inhibition for the detection of bovine respiratory syncytial virus.

Age of animals	Collected samples	Positive samples	%
2 – 3 years	43	9	20.9
3 – 4 years	55	11	20
4 – 5 years	21	6	28.6
Total	119	26	21.8

Table 6. Results of ELISA for the detection of antibodies against bovine respiratory virus.

Age of animals	Collected samples	Positive samples	%
2 – 3 years	43	13	30.2
3 – 4 years	55	20	36.4
4 – 5 years	21	10	47.6
Total	119	43	36.1

DISCUSSION

Commensal bacteria present in the respiratory system may cause diseases when animals are subjected to stress factors *(27)*, also virus are important in the bovine respiratory disease complex, they play role by reducing the ability of the immune system of cattle to deal with the bacterial invaders that may result in pneumonia *(28)*. Examination of 119 lung samples from calves revealed that 88.24% of them (Table 1) were harbored pathogenic bacteria such high incidence of isolation was also reported by *(29,30)*. High percentage of mixed culture were 31.43%, the incidence of isolation of one organism from pneumonic lungs were (68.57%) nearly similar the results were reported *(22)* (Table 1) .These organisms isolated in single form as *E.Coli* (20%), *Pasteurella multocida* B;6 (14.33%) and *Staphylococcus aureus* (30.48%) or coupled *E.Coli* with *Pasteurella multocida* B;6 were (11.43%), *Staphylococcus aureus* with *E.Coli* (10.48%) and *Staphylococcus aureus* with *E.Coli* with *Pasteurella multocida* B:6 were 30.47% in (Table 2). These results were agreement with *(29)* who isolated *E.Coli* (20%) but differ in isolation both *Pasteurella multocida* (2%) and *Staphylococcus aureus* (53.5%), while *(21)* who isolated *E.Coli (8.69%)* differ in the present investigation and agreement with isolated *Pasteurella multocida* (13.04%) and *Staphylococcus aureus* (30.47%) from lungs cattle.

Mixed infection in this investigation mainly between *E.Coli* and *Pasteurella multocida* B:6 (11.43%), *Staphylococcus aureus* with *E.Coli* (10.48%) and both with *Pasteurella multocida* B:6 both are observed 30.47%, *E.Coli* with *Pasteurella multocida* known as environmental pathogenic microorganisms and are frequently encourented in both upper and lower respiratory tract specially in animals housed at bad hygienic condition *(28,31,32)*.The presence of mixed infection mainly *Staphylococcus aureus* with *E.Coli* and other organisms indicate the complexity of the situation. The *Staphylococcus aureus* may predispose the dairy herd to infection by Coliform organisms or other pathogens *(33)*.

Sensitivity test of the antibiotic was carried on the 3 different types of bacterial isolates which represent the main causative agents of bacterial pneumonia in slaughtered calves (Table 3) most of the bacterial isolates were highly sensitive to Enerofloxaccine

,Gentamycine and Streptomycine while most strains resistant to Clindospectine, the results supported the previous findings of ***(31,34).***

Bovine respiratory syncytial virus (BRSV) is associated with outbreaks of acute respiratory disease in cattle. Pulmonary lesions described in calves in spontaneous outbreaks of respiratory tract disease associated with BRSV have included bronchitis, bronchiolitis and interstitial pneumonia with development of interstitial emphysema in fatal cases ***(35).***

The lung samples were directed to virus isolation and identification by virus neutralization test, in addition to detection of viral antigen by using haemagglutination inhibition test. While serum samples were directed to detection of antibodies against BRSV using of ELISA test.

The results of virus isolation on MDBK cells indicate that 18 (15%) out of 119 pneumonic lungs are positive for the presence of BRSV after 3-5 passages. The isolated virus was identified as a bovine respiratory syncytial virus using of virus neutralization test with known hyperimmune serum against BRSV (Table 4).

The percentage of identified isolated virus (15%) was considered low because BRSV is one of the complex respiratory disease among cattle. Tissue samples containing high concentration of BRSV antigens frequently do reproduce the virus in cell cultures. Several factors are involves, mainly high virus liability ***(36,37)***. Virus isolation was achieved from collected samples from imported cattle the higher sensitivity of imported cattle to BRSV due to stress factor ***(18)***.

The recorded result was in agreement with the previous study ***(18,19)***.

BRSV in 2.2% ***(38)*** and 3% ***(12)*** from of lung samples collected from diseased cattle. These differences could be attributed to the type of cell culture or the severity of infection and subsequently to the virus titer in the collected samples.

The detection of BRSV antigen was carried out using of haemagglutination inhibition test on all collected 119 lung samples. The obtained results revealed that 26 (21.8%) out of 119 collected samples were give positive results (Table 5).

The obtained results were in agreement with ***(18,39)*** who detected BRSV antigen in 22.4% and 21.6% of collected lung samples respectively.

However, the record results were less than those obtained by ***(40)*** who detected BRSV antigens in 65.6% out of collected lung samples tested by HI test.

Although, it is herd that for us to compare the results of the used test due to the difference in number and test used. But it seems that haemagglutination inhibition test is a specific serological technique acting by using of specific known hyperimmune serum against known virus. If the haemagglutinating activity not appear, it means that the examined virus is specific to the known huperimmune serum. ***(41)***.

Detection of antibodies in the collected serum samples from slaughtered animals and tested by ELISA test revealed that 43 (36.1%) out of 119 serum samples were positive for the presence of antibodies against BRSV (Table 6). The obtained results were in agreement with ***(19,42)*** who detected antibodies against BRSV in 36.4% and 37.5% respectively in the collected serum samples from diseased cattle and tested by ELISA test.

Higher percentage were recorded by ***(43,44)*** who reported antibodies against BRSV in the collected serum samples collected from affected cattle and tested by ELISA with percentages of 85% and 54% respectively.

On contrary, ***(45,46)*** were reported antibodies against BRSV in a low percentages (9.8%) and (10.5%) respectively in the collected serum samples from diseased animals and tested by ELISA test.

It is noticed that the differences between ELISA test results in the present study and the other recorded results had been mentioned in

this research may be attributed to the seasonal variation and the variation in the immunostatus of the affected animals.

In conclusion, the present study reported that BRSV infection is present in Sharkia Governorate among cattle especially the young ages. This viral infection play an importent role in reducing the ability of immune system to deal with commensals bacteria in the upper respiratory tract which attack the lung resulting in pneumonia. Although the lower percentage of percentage viral infection associated with winter season. Serological investigation showed that ELISA technique is the specific serological test in routine detection of BRSV.

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الملخص العربي

المسببات البكتيرية والفيروسية للالتهابات الرئوية فى عجول التسمين المذبوحة في محافظة الشرقية

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أجريت هذه الدراسة على ١١٩ عينه من رئات و ١١٩ عينة أمصال من عجول بها أعراض التهابات رئوية مذبوحة في مجازر محافظة الشرقية. أخذت هذه العينات للفحص البكتيريولوجى والفحص الفيرلوجى. تم زراعة هذه العينات على أوساط غذائية مختلفة وفران بيضاء وأختبار تلازن الدم الغير مباشر وأختبار ألأجار جل الترسيبى للباستيريلاللعزل البكتيريولوجى وقد أوضحت نتائج زرع هذه العينات على عزل ١٠٥ (٨٨,٢%) عترة بكتيرية وكانوا كالتالى: ميكروب الباستيريلا ب:٦ (٣٣و٣%) وميكروب القولون العصوى (٢٠%) وميكروب العنقودى الذهبى (٩٢و١٤%) وتم عزل ميكروب الباستيريلا ملتوسيدا ب:٦ مع ميكروب القولون العصوى (٤٣و١١%) وميكروب العنقودى الذهبى مع القولون العصوى (٤٨و١٠%) وميكروب العنقودى الذهبى مع ميكروب القولون العصوى مع ميكروب الباستيريلا ب:٦ (٤٧و٣٠%) . وتم أجراءأختبار الحساسية لكل من العترات البكتيرية المعزولة ووجدان معظم العترات المعزولة شديدة الحساسية لكل من أنروفلوكساسين وجنتاميسين وأستربتوميسين وقليل الحساسية لكلندوميسين. بالنسبة للفحص الفيروسي , تم عزل الفيروس التنفسي التنخمي للأبقار على خلايا الزرع النسيجي (MDBK) و التعرف على الفيروس باستخدام الاختبار التعادلي للفيروس في ١٨ (١٥%) من عينات الرئات المجمعة. كذلك تم الكشف عن الأنتيجين الخاص بالفيروس التنفسي التنخمي للأبقار في ٢٦ (٢١,٨%) و ذلك باستخدام اختبار منع تلزن الدم على عينات الرئات المجمعة من الحيوانات المصابة. إضافة إلى ذلك , تم الكشف عن الأجسام المناعية المضادة للفيروس في ٤٣ (٣٦,١%) و ذلك باستخدام اختبار الإليزا على أمصال الحيوانات المصابة و التي تم تجميعها.