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Conventional Detection of Bacterial and Mycotic Infection in Pneumonic Lungs of Cattle Associated with Severe Bovine Respiratory Disease

Ahmed, M.E. Yousef[1]*, Ahmed, M.A. Zaitoun[2]

[1]*Infectious Diseases, Dept. Animal Med., Faculty of Vet. Med. Sohag Univ., 82524*
[2]*Infectious Diseases, Dept. Animal Med., Faculty of Vet. Med. Assiut Univ., 71515*

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Abstract: During the period of investigation from July 2021 to April 2023, a total of 650 cattle were clinically inspected. Eighty diseased cattle with severe signs of Bovine Respiratory Disease (BRD) were clinically examined. These cases did not respond to antibacterial and anti-inflammatory treatments under Egyptian field conditions. A post-mortem examination of the pneumonic lungs of emergency slaughtered and recently succumbed cases showed fibrosis, caseonecrotic bronchopneumonia, and multiple abscess formations. Pneumonic lung samples were collected for bacterial and mycotic culturing. The frequent distribution of the respiratory pathogens isolated from the pneumonic lungs of necropsied cases (n = 63) were *Trueperella pyogenes* (*T. pyogenes*) 90.48% (14/63), *Mannheimia haemolytica* (*M. haemolytica*) 85.72% (54/63), *Staphylococcus aureus* (*Staph aureus*) 85.72% (54/63), *Pasteurella multocida spp. multocida* (*P. multocida*) 9.52% (6/63), βeta-hemolytic Streptococci (βHS) 9.52% (6/63), *Escherichia coli* (*E. coli*) 9.52% (6/63), *Aspergillus flavus* (*A. flavus*) 9.52% (6/63), and *Aspergillus niger* (*A. niger*) 4.76% (3/63). The synergistic situation between bacterial and mycotic infections aggravated the clinical signs of the disease and made treatment impossible.

Keywords: *Aspergillus flavus*, *Aspergillus niger*, Bacteria, BRD, and *Trueperella pyogenes*.

INTRODUCTION

BRD remains a huge global economic burden for both the dairy and beef industries causing severe economic losses as, 70%- 80% morbidity and 40%- 50% mortality, major cause of deaths. Moreover, loss of production, drop of milk production till complete cessation, treatment costs and negatively impact growth, reproductive performance, carcass weight, marbling and other carcass value factors (Griffin *et al.*, 2010 and Schaffer *et al.*, 2016).

Three interdependent factors—infectious agents, a weakened host immune system, and environmental and managerial factors—contributed to the development of BRD (Grissett *et al.*, 2015). Consequently, the disease's diagnosis is difficult because there are numerous potential causes (Fulton and Confer, 2012).

There are enormous pathogens encountered as pneumo-pathogens including viruses, Mycoplasma, bacteria and Fungus (Sayed El-Ahl *et al.*, 2014 and Guzman and Taylor, 2015). However, viral pathogens usually play an outstanding role as primary pathogen for BRD due to lung damage and immune dysfunction allowing the secondary infections (e.g. bacteria, and fungus) inducing severe pneumonia followed by death (Hanon *et al.*, 1998 and Pastrana *et al.*, 2022).

The predominant bacterial pathogens isolated from pneumonic lungs of cattle are *M. haemolytica, T. pyogenes, Staphylococcus aureus, S. pyogenes, E. coli, and P. multocida* are (Sayed and Zaitoun, 2009).

Sayed El-Ahl *et al.* (2014) reported that the main isolated yeasts and molds from cattle showing pneumonia with or without nasal discharge reared in private farms at El-Menoufea Governorate, North-Egypt were *Aspergillus fumigatus, Aspergillus niger, Aspergillus candidus, Aspergillus flavus, Penicillum Sp.* and *Candida Sp.*

Therefore, the current work was carried-out to reveal-up the most common isolated bacterial and mycotic pathogens from pneumonic lungs of recently succumbed and/or emergency slaughtered cattle with previous history of sever BRD.

MATERIALS AND METHODS

Ethical Approval

All experimental protocols that were accepted by the committee's consideration of animal research were carried out by the Faculty of Veterinary Medicine at Sohag University.

ANIMAL AND AREAS OF STUDY

Six hundred fifty cattle underwent clinical inspection over the investigation's interval from July

***Corresponding Author:** Ahmed, M.E. Yousef

2021 to April 2023. These animals came from four governorates in Egypt: Sohag, Assiut, and Cairo (local breed cattle), and Abu-Simbel (imported cattle of African breed). The clinically examined cattle were divided into two categories. Group I consisted of 300 local breed cattle spread across the governorates of Sohag, Assiut, and Cairo. The second group included 350 imported cattle of the African breed (n = 350). Sixty-three diseased cattle with severe signs of respiratory manifestations were recently succumbed or emergency slaughtered inside and/or outside slaughterhouse. These cases did not respond to commercial antibacterial drugs under Egyptians field conditions.

SAMPLES

Sixty-three tissue specimens were taken from pneumonic lungs after gross necropsy for animal's emergency slaughtered inside and/or outside slaughterhouse or recently succumbed with severe respiratory signs was carried out as described by (King *et al.*, 2014).

Samples were identified to show the serial numbers, animal species, breed, age, sex, date of sampling and other observations. The tissue specimens of pneumonic lungs were immediately immersed in screw-capped bottle containing brain-heart infusion broth (BD) supplemented with 5% heated-inactivated horse serum (Sigma- Aldrich®, St. Louis, Missouri, U.S.A., catalog no.: H1270 Sigma) for bacteriological examination and another tissue specimens from the same lungs were put into a clean, dry and sterile polyethylene bags under a septic condition for mycotic investigation. All samples were put into tank double wall ice box and brought to Laboratory of Infectious Diseases and the Central Laboratory, Faculty of Veterinary Medicine, Sohag University, Egypt with minimal delay.

For bacterial isolation the broth tube was incubated at 37ºC for 24 – 48 hours and thereafter platted onto the following media 5% sheep blood agar, Egg-yolk-potassium tellurite-Baird Parker agar, Streptococcus selective agar (SSA) (BD Co.). The cultured plats were incubated aerobically for 24 – 48 hours with exception of SSA's plats incubated in low-oxygen tension environment (candle Jar incubation). Post incubation the suspected colonies were picked-up and sub-culturally purified.

The Gram-positive purified strains were identified morphologically and biochemically based on the criteria illustrated by Quinn *et al.* (1994). This criteria includes morphological characterizations of colonies particularly on selective media, hemolysis on blood agar, Gram staining with bacterial morphology characteristics, growth on mannitol salt agar and MacConkey's agar media, catalase, oxidase, wet-mount-motility, Rabbit's coagulase test, and CAMP and reserve CAMP reactions.

On the other hand, the Gram-negative colonies were cultured on MacConkey's agar plates and Eosin-methylene blue agar plates. Suspected colonies on MacConkey's media were inoculated into T.S.I. agar by stabilizing the butt first and then streaking slant, followed by incubating at 37ºc for 24-48 hours. The interpretation of the reaction to T.S.I. was performed according to Quinn *et al*. (1994). Final identification for the isolates was carried out by using the following tests; Indole test, Methyl red test, Voges proskauer test, Citrate utilization test by kosser' s medium, Gelatin liquefaction, and Fermentation of sugars.

The following tests were used for the isolates' final identification: the methyl red, indole, and voges proskauer tests; the citrate utilization test using Kosser's medium; the liquefaction of gelatin; and the fermentation of sugars. For mycotic isolation, Czapek dox agar (Biolife italiana®, Italy, Catalog no.: 4013602) was used. The specific methods for the identification of the individual pathogenic fungi were carried out according to (Quinn *et al*., 1994). Tissue samples were taken and distributed onto two sets of Czapek dox agar plates after surface decontamination scorching with a hot spatula and incubated for 7 days at 25° C and 37° C, respectively. The medium was made more selective by adding chloramphenicol (antibacterial). Culture media was streaked with inoculation from the pneumonic lung as for bacterial cultures. Sub-culturing fungal colonies by an inoculation loop made slightly moist and sticky by pushing it into a portion of sterile agar. Microscopic examination of fungal colonies with dissecting microscope carried out according to Quinn *et al*. (1994).

For fungal identification, Wet mount method, the lactophenol cotton blue stain method the slide culture technique, morphology of the colony and type of pigmentation with the microscopic appearance of the fruiting heads and spores was done according to Quinn *et al*. (1994).

RESULTS

Clinical examination of cattle with severe BRD showed pathognomonic signs; dullness, lethargy, extended head and neck, ear dropping, abduction forelimb, dilated nostrils, bilateral tenacious yellow sticky nasal discharge, oral breathing, lingual hypotonia, and congested conjunctival mucous membrane (Fig. 1a, b, c, d).

Necropsy examination of recently succumbed animals and/or emergency slaughtered animals inside the abattoir and/or outside the abattoir with sever signs of BRD elucidated acute lobar pneumonia with severely congested lung tissue, yellowish exudate and pleural adhesions as fibrin depositions (Fig. 2a). Acute fibrinous bronchopneumonia (lobar pneumonia) with consolidation and hard texture of lung lobe with fibrin deposition and pleural adhesions with hemorrhages fill the thoracic cavity (Fig. 2b). that interstitial lobar

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pneumonia with diffuse distribution, heavy, rubbery texture and 'port-wine' staining of both right and left lung (Fig. 2a). Cranioventral dark red to mahogany consolidation with firm texture of cranial lobe lung 30% of pulmonary parenchyma with yellowish-white to greenish-white raised nodules and normal pulmonary parenchyma in 70% of the caudal and cardiac lobes were located (Fig. 2c). Cut surface, dark red consolidated firm texture and greenish-white purulent exudate in the bronchioles (Fig. 2d).

Bacterial isolation from pneumonic lung tissues of recently succumbed or for animal's emergency slaughtered inside and/or outside slaughterhouse with severe BRD signs revealed that were often distributed as follows:, *T. pyogenes 90.48%(14/63), M. haemolytica* 85.72% (54/63), *Staph aureus* 85.72% (54/63), *P. multocida spp. multocida* 9.52% (6/63), *βHS* 9.52% (6/63), *E. coli* 9.52% (6/63).

Nine (14.28%) of 63 necropsied cases were fungus-culturing positive (Fig. 3). The morphology of the colony and type of pigmentation, as well as the microscopic appearance of the fruiting heads and spores were examined, and the presence of *Aspergillus niger* and *Aspergillus flavus* infections was discovered with 4.76% (3/63) and 9.52% (6/63) respectively (Table 1).

Table 1. The most common isolated respiratory pathogens from necropsied cases

Pathogens	Nr. of cases (63)	%
Staph aureus *Trueperella pyogenes* *Mannheimia haemolytica*	28	44.45%
Mannheimia haemolytica *Staph aureus* *Trueperella pyogenes*	26	41.27%
Aspergillus flavus *Pasteurella multocida spp multocida* *β haemolytic streptococci* *E.coli*	6	9.52%
Aspergillus niger *Trueperella pyogenes*	3	4.76%

Fig. 1: Fig.1 Pathognomonic signs of BRD. A) 3.5 years Friesian cow with epiphora, dilated nostrils and bilateral tenacious yellow sticky nasal discharge. B) Dullness, lethargy with accumulation of flies on the snout of 2.5 years Holstein Friesian

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bull with bilateral tenacious sticky nasal discharge, congested conjunctival mucous membrane and epiphora. C) Cough test in 8 months mixed breed cow with sever BRD. Ear dropped oral breathing lingual hypotonia, abduction forelimb. D) Extended head and neck, dilated nostrils and oral breathing, lingual hypotonia congested conjunctival mucous membrane in 4 years dairy cow.

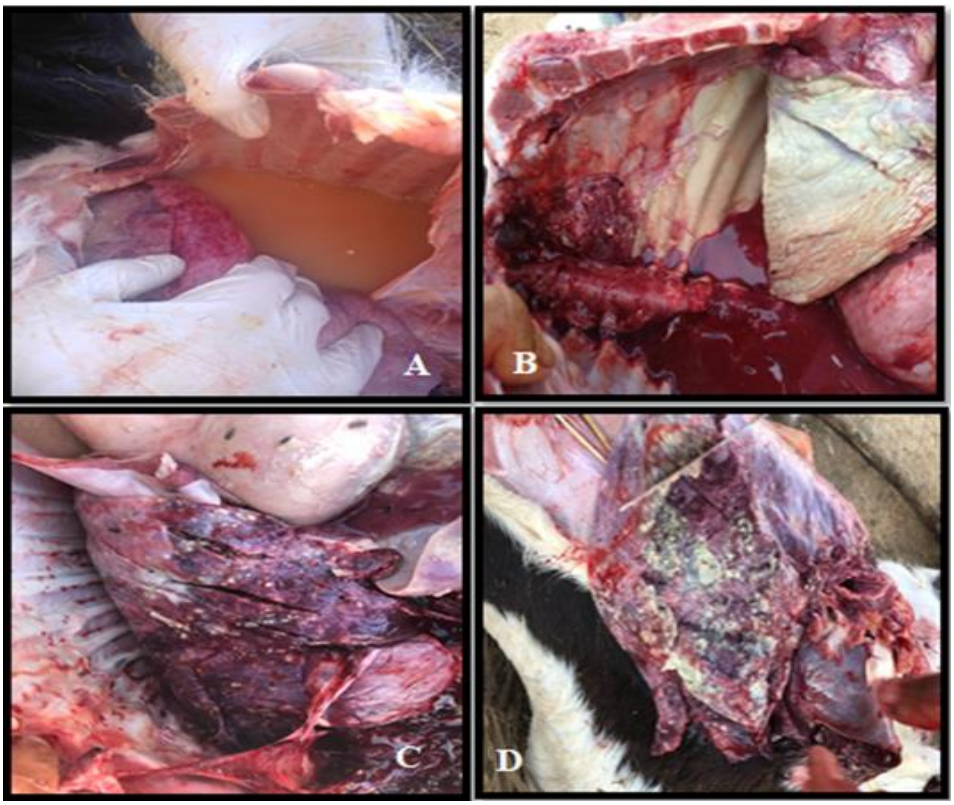

Fig. 2: Post-mortem findings of the necropsied cases. A) Yellowish exudate and pleural adhesions (parietal and visceral pleura) as fibrin depositions and cranioventral purple, dark red consolidated severely congested lung tissue in recently succumbed neonatal Holstein calf with acute lobar pneumonia. B) Acute fibrinous bronchopneumonia (lobar pneumonia) with consolidation and a hard texture of the lung lobe with fibrin deposition and pleural adhesions with hemorrhages filling the thoracic cavity (hemothorax). C&D) Suppurative bronchopneumonia (lobular pneumonia) of a recently succumbed neonatal Friesian calf with signs of respiratory distress. C) Cranioventral dark red to mahogany consolidation with firm texture of the cranial lobe of the lung 30% of pulmonary parenchyma with yellowish-white to greenish-white raised nodules and normal pulmonary parenchyma 70% of caudal and cardiac lobes. D) Cut surface dark red consolidated firm texture and greenish white purulent exudate in the bronchioles.

DISCUSSION

BRD is a devastating syndrome for cattle and has a major impact on the health and economic performance (Griffin, 1997) causing severe economic losses as, 70%- 80% morbidity, 40%- 50% mortality, major cause of deaths, loss of production, treatment coasts and negatively impact growth, reproductive performance, carcass weight, marbling and other carcass value factors (Larson, 2005; Griffin *et al.*, 2010 and Schaffer *et al.*, 2016).

BRD is a multifactorial causes involving infectious agents, compromised host immune system, and environmental and managmental factors including stocking density, cattle mixing, dust, transport time, nutritional changes and other stressors predispose susceptible cattle to the development of BRD (Dyer, 1982 and Grissett *et al.*, 2015). Environmental and management factors play significant roles in the prevalence of pneumonia especially bad hygienic measures, accumulation of fecal matter and urine beneath animals with poor ventilation lead to increase ammonia level which has worsen effect on animal respiratory system and must be considered in a holistic approach to reduce BRD (Snowder *et al.*, 2005 and Snowder, 2009). Mac Vean *et al.* (1986) indicated that dust particles between 2.0 to 3.3 µm in diameter impacted BRD incidence.

Transportation was the most universally accepted non-infectious risk factor for pneumonia and led to shipping fever. The distance and/or time in transit had a positive association with morbidity of BRD (Snowder *et al.*, 2006 and Snowder, 2009).

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Clinical examination of cattle with severe respiratory distress showed pathognomonic signs; dullness, lethargy, extended head and neck, ear dropping, abduction forelimb, dilated nostrils, bilateral tenacious yellow sticky nasal discharge, oral breathing, lingual hypotonia, and congested conjunctival mucous membrane similar clinical abnormalities were described by (Kelly, 1974 and Sayed *et al*., 2002).

Necropsy examination of necropsied cases elucidated acute lobar pneumonia with severely congested lung tissue, yellowish exudate and pleural adhesions as fibrin depositions. Acute fibrinous bronchopneumonia (lobar pneumonia) with consolidation and hard texture of lung lobe with fibrin deposition and pleural adhesions with hemorrhages fill the thoracic cavity. Cranioventral dark red to mahogany consolidation with firm texture of cranial lobe lung 30% of pulmonary parenchyma with yellowish-white to greenish-white raised nodules and normal pulmonary parenchyma in 70% of the caudal and cardiac lobes were located. Cut surface showed, dark red consolidated firm texture and greenish-white purulent exudate in the bronchioles similar necropsy findings were elucidated by (Zachary, 2017 and Haydock *et al*., 2023).

Bacterial examination showed that *T. pyogenes* followed by *M. haemolytica*, *Staph aureus*, *P. multocida spp. multocida*, *βHS*, and *E. coli* were the most common isolated bacterial respiratory pathogens (Haydock *et al*., 2023). The percentage of frequent isolation of the isolated bacterial pathogens from the necropsied cases *T. pyogenes*, *M. haemolytica*, *Staph aureus*, *P. multocida spp. multocida*, *βHS*, and *E. coli* were *90.48%*, 85.72%, 85.72%, 9.52%, 9.52%, 9.52%, respectively. Francis and Ameh (2015) isolated *Staph aureus* with 18.67%; *Streptococcus pneumonia* with 12%; *Pasteurella multocida* with 11.33%; *E. coli* with 10% and *T. pyogenes* (formely Corynebacterium spp) with 8% from pneumonic lungs of cattle slaughtered at Maiduguri Municipal abattoir in Borno State, Nigeria. Sedeek and Thabet (2001) isolated 20 bacterial isolates from pneumonic lungs of cattle which were suffered from pneumonia in Assiut Governorate, Egypt and they found that *Staph aureus* was isolated with 30%, *E. coli* with 5% and *Streptococcus pyogenes* with 15%. In another study Sayed and Zaitoun (2009) found that the percentage of isolation of the bacterial pathogens from the examined pneumonic lungs of feedlot buffalo-calves suffering from pneumonia and slaughtered at Assiut abattoirs, Assiut Governorate, Egypt were *Staph aureus* 22.43%; *E. coli* 18.22%, *Pasteurella multocida* 15.89%, *Streptococcus pyogenes* 5.61%, and *Trueperella pyogenes* 3.74%.

Infection with the pathogenic fungi can arise from ingestion of the contaminated feed or water with fungi or inhalation dust particles came from contaminated soil with fungi (Seyedmousavi *et al*., 2015). Pulmonary aspergillosis plays a significance impact in cattle industry (Desoubeaux and Cray, 2018). Khalifa *et al*. (2022) mentioned that the frequency of occurrence of toxigenic fungi in animal feedstuff samples were *A. flavus* recording the highest percentage (65.56%) followed by *A. niger* (50%). *A. flavus* is the second leading cause of aspergillosis pathogen in animals after *A. fumigatus* and has a virulent factors makes it pathogenic causing lung damages of animals (Lan *et al*., 2018). *A. flavus* is a pathogenic fungus that can produce carcinogenic and toxic aflatoxins that are a serious and has a harmful effect to animals (Nierman *et al*., 2015 and Yang *et al*., 2021).

In the present study, 9 (14.28%) of 63 necropsied cases were fungus-culturing positive. *A. niger* and *A. flavus* infections was discovered with 4.76% (3/63) and 9.52% (6/63) respectively. Carter *et al*. (1973) isolated *Aspergillus spp.* from bovine systemic mycoses especially from abortion and pneumonic cases by culturing methods. Sayed El-Ahl *et al*. (2014) revealed that the main isolated yeasts and moulds from 50 pneumonic lungs tissues collected from private farms at El-Menoufea governorate, Egypt were *A. fumigatus*, *A. niger*, *A. candidus*, *A. flavus*, *Penicillum Sp*. and *Candida Sp*. with the percentage 8% , 8% , 0% ,8% , 24% and 40%, respectively.

There are enormous pathogens encountered as pneumo-pathogens including viruses, mycoplasma, bacteria and fungi (Carter *et al*. 1973, Bryson, 1985 and Guzman and Taylor, 2015). However, viral pathogens usually play an outstanding role as primary pathogen for BRD due to lung damage and immune dysfunction allowing the secondary infections by bacteria and fungi (Gaudino *et al*., 2022). A number of studies had demonstrated a synergistic role of respiratory viruses in cattle (Kirchhoff *et al*., 2014 and Pastrana *et al*., 2022).

Good control to BRD depends mainly on vaccination program of cattle against respiratory pathogens in parallel with good management and hygienic measures; high quality feed, avoid deprivation cattle from feed and water, improvement environmental condition and avoiding stress factors, long transportation, overcrowdness, bad sanitary condition by continuous cleaning and removing fecal matter and urine and good ventilation to avoid increasing level of ammonia production (Gorden and Plummer, 2010). The calf's resistance to BRD was significantly influenced by the maternal antibodies that were passively acquired (Bryson, 1985).

CONCLUSION

BRD is a devastating syndrome for cattle and has a major impact on their health and economic performance. Pneumonia is a multifactorial cause involving infectious agents, compromised host immune systems, and environmental and managmental factors. Bacterial-fungal mixed infections occur after immune suppression by viral or mycoplasma infection. Fungal

***Corresponding Author:** Ahmed, M.E. Yousef

isolation in the diagnosis of BRD is a missed diagnostic pathogen and must be kept in mind during diagnosis. Cattle immunization against respiratory infections, along with proper management and hygienic practices, are the essential components of an effective BRD control program.

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