Investigation of Bovine Respiratory Disease Complex in Egypt with Emphasis on Some Viral and Bacterial Pathogens

Kawther S. Zaher[1], Sohier M. Syame[1], Hanan M. Elhewairy[2] and Hanan S.H. Marie[1]

[1]Microbiology and Immunology Department, National Research Center (NRC), Dokki, Cairo, Egypt.
[2] Immunology Department, Animal Health Research Institute, Dokki, Egypt.
zaherkus@yahoo.com

Abstract: Bovine Respiratory Disease Complex (BRDC) caused by various viral and bacterial agents. The current study is aiming to investigate some of the causative agents which considered being a major problem such as Bovine herpes virus-1(BHV-1), parainfluenza-3(PI-3), Bovine respiratory syncytial virus (RSV) and Bovine viral diarrhea (BVD), Mannheimia haemolytica (M. haemolytica) and Pasteurella multocida (*P. multocida*). Blood samples were taken from 227 mature buffalo cows at Al Sharquia and Al Daqhaliya Governorates of Egypt. Dot ELISA, PCR and electron microscopy were performed to diagnose the viral agent and the nasal swabs were plated onto 5% sheep blood and MacConkey's agar for 24 hrs at 37°C under aerobic conditions. The plates were examined for growth, colony morphology, colour and odour. Bacterial identification was performed by biochemical testing to identify *P. multocida* and/or *M. haemolytica.* Results diagnosed BRDC in 40.53% buffalo cows. Infected animals showed clear clinical signs of the disease only in 25.99% animals, while 66.96% of these animals did not show any signs. 16.74% of animals showed signs of BRDC revealed more than one sign. Dot ELISA revealed that 14.54% of serum and nasal swabs samples from apparently healthy Buffalo-cows were positive for BRDC. However, 100% of the samples collected from animals showing the disease symptoms contained BRDC. PCR was confirmatory test and detected higher incidence as compared to Dot ELISA in both BRS and BVD samples and significant ($P< 0.05$) incidence in BHV-1 and PI-3 samples 15 and 29% in PCR compared to 11 and 25% in Dot-ELISA, respectively. Electron microscopic examination showed the same morphology and approximate size of each virus. The bacterial inoculation isolated *Pasteurella* (n = 32) and *Mannheimia* (n = 15), while biochemical testing identified *P. multocida M. haemolytica.* The most observed result was the frequent association between BVD with *M. haemolytica* (n = 7) followed by PI-3 with *P. multocida* (n = 6), BHV-1 with *P. multocida* (n=5), BHV-1 with *M. haemolytica* (n= 5), BRSV with PI-3 (n = 5) and the BRSV-PI-3 (n = 5). In the case of triple infections (15/92), the interaction between BRSV- PI-3 and *P. multocida* was observed in four cases, then the association between BHV-1 and PI-3 *P. multocida* (n=2), while association of BRSV-*P. multocida* and *M. haemolytica* as well as PI-3- *P. multocida- M. haemolytica* and BRSV- BVD - *P. multocida* was observed once.

[Zaher, K.S., Sohier M. Syame, Hanan M. Elhewairy and Hanan S.H. Marie. **Investigation of Bovine Respiratory Disease Complex in Egypt with Emphasis on Some Viral and Bacterial Pathogens.** *Life Sci J* 2014;11(6):56-62]. (ISSN:1097-8135). http://www.lifesciencesite.com. 8

Keywords: BRDC, BHV-1, PI-3, BVD, *P. multocida, M. haemolytica*

1. Introduction

Diseases of the respiratory tract amount to substantial economic loss in the cattle industry every year. They are often referred to as Bovine Respiratory Disease Complex (BRDC) caused by stress, virus agents, and bacterial agents. These agents often produce mild clinical to severe clinical signs and death within 24 to 36 hours or may cause permanent lung damage as fibrosis, adhesions and/or abscesses, in chronic cases, which will impact performance. That is why early recognition and treatment of BRDC are so important (Fulton *et al.,* 2009; Intisar *et al.,* 2009; Regev-Shoshani *et al.,* 2013; Torres *et al.,* 2013; Kirchhoff *et al.,* 2014).

Bovine herpesvirus-1 (BHV-1), also known as Infectious Bovine Rhinotracheitis (IBR) virus, family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus*, is one of the BRDCV of cattle and buffaloes associated with respiratory, enteric, ocular, nervous systems and genital infections. The virus consists of a relatively large double-stranded, linear DNA genome encased within an icosahedral protein cage called the capsid, which is wrapped in a lipid bi-layer called the envelope (Kamaraj *et al.,* 2009; Crook *et al.,* 2012).

Bovine parainfluenza-3 (PI3) has been associated with both acute and chronic pneumonia in cattle. It is an enveloped, non-segmented, negative-sense RNA virus within the genus *Respirovirus* and the family *Paramyxoviridae*. Infection with PI3 is often concurrent with BHV1 and/or BVDV infection. (Ghram *et al.,* 1989) observed that calves infected with BHV1 and PI3 developed clinical signs including fever, cough, nasal and ocular discharges.

Bovine respiratory syncytial virus (BRSV) is an enveloped single-stranded negative sense RNA which

belongs to family *Paramyxoviridae* and genus *Pneumovirus*. It has been established as a common pathogen in respiratory disease and has been demonstrated to interact with bacterial pathogens in establishing pneumonia in cattle. BRSV occurs within the cattle population around the world. It is not commonly diagnosed not because it does not exist but because it is difficult to isolate (Stott *et al.,* 1980; Trigo *et al.,* 1984).

The role of bovine viral diarrhea virus (BVDV) in shipping fever pneumonia in feedlot cattle remains controversial in spite of decades of research. BVDV is an enveloped, linear positive sense, single-stranded RNA virus which belongs to the family *Flaviviridae* within the order *Pestivirus*. It has been reported to be the virus most commonly isolated from pneumonic lungs of cattle. In some studies, infection with BVDV followed by inoculation, after several days, with *M. haemolytica* has resulted in severe pneumonic disease (Martin *et al.,* 1989).

The severe pneumonic damage characterized by pulmonary invasion of *Pasteurella multocida* (*P. multocida*) and *Mannheimia haemolytica (M. haemolytica)* and other bacteria is associated with the production of virulence factors which facilitate colonization of the lower respiratory tract (Whiteley *et al.,* 1992). Production of neuraminidase and neutral protease may enhance the bacterium's ability to adhere and colonize the respiratory epithelium (Yoo *et al.,* 1995). Lung injury characterized by vascular damage, excess fibrin effusion, and neutrophil infiltration results from the host's response to leukotoxin produced by these Gram-negative organisms (Breider *et al.,* 1988; Yang *et al.,* 1989; Ackermann and Brogden, 2000; Torres *et al.,* 2013).

The current study aims to investigate BRDC and to develop a simple, sensitive, specific, rapid diagnosis of the viral and bacterial causative agents in Egyptian cows and buffaloes and comparing the assay with the conventional methods of isolation and identification involved in BRDC.

2. Material and Methods

This work was performed in The National Research Center (NRC) in the period between December 2012- June 2013.

Animals:

A total number of 227 mature buffalo cows raised at areas deprived from general services (as periodic vaccination) at Al Sharquia and Al Daqhaliya Governorates of Egypt were investigated. These animals were 2- 6 years old, kept in small holder farms and fed on Egyptian clover during December to May and concentrate, crop residues and rice straw during other months of the year, there is no regular system of vaccination.

Experimental design:

A complete case history and owner complaint were recorded for each animal. Animals were clinically examined and samples were taken from animals showing lethargy or depression, reduced feed intake, fever, increased respiratory rate, dyspnea, with or without nasal, ocular discharge and pneumonia.

Samples collection:

Blood Samples were collected from the jugular vein (heparinized and non heparinized blood); and nasal swabs from 227 buffalo-cows in Al Sharqiya and Al Qalyoubiya governorates of Egypt.

Preparation of samples:

Heparinized blood was centrifuged at 3000 rpm for 5 min and the puffy coat was collected. While in non heparinized blood the blood sample was centrifuged at 2500 rpm for 5min and serum was collected. Nasal swabs were immersed in PBS containing 100 µg/ml penicillin, and 100 µg/ml streptomycin.

Virological assay:

All virological tests were carried out using a locally isolated BHV-1, PI-3, BRSV and BVD and its antisera, compassionately obtained from the Veterinary Serum and Vaccine Research Institute, Abbassia, Egypt.

A-Dot ELISA test: The test was performed as outlined by White and Fenner (1986).

B- Monoplex PCR and RT-PCR

Commercial kit was used for extraction of both DNA and RNA (Qiagen Co., Valencia, CA, USA) and the steps were performed according to manufacturer's protocol. For RT-PCR, it was performed by means of QIAGEN One-Step RT-PCR Kit (QIAGEN, GmbH, Germany). While for DNA virus PCR was performed by Qiagen PCR Kit (QIAGEN, GmbH, Germany).

Primers for BHV-1:

The sequences of the oligonucleotide primers used in the monoplex PCR assay were based on the published BHV-1 glycoprotein gI sequence. The primers used in PCR were 5' TAC CTG CGC AGC GGG CGC 3' and 5' CTT CGA TCA CGC AGT CGC TCA 3' were identical to the sense and antisense strands from nucleotides 651 to 669, and 1273 to 1294, respectively. The PCR was performed according to Van Engelenburg *et al.* (1993).

Primers for PI-3:

The sequence of utilized primers was as follows: Sense 5' CAT TGA ATT CAT ACT CAG CAC 3', while the antisense 5' AGA TTG TCG CAT TTA GCC TCA 3'.

The primers were chosen from conserved site F gene sequences of bovine PIV3 in the EMBL sequence

bank. RT-PCR was applied according to Lyon *et al.* (1997); Zhu *et al.* (2011).

Primers for BRSV:

RT-PCR was applied and a set of primers, derived from the published sequence of RSV G gene (Gene Bank accession # L08470) was synthesized and used for amplification of a 542-bp G gene fragment of RSV. One primer 5' AGC CCT AGC AAT GAT AAC 3' representing bases 147–164 of RSV G gene was used for complementary (cDNA) synthesis. The second primer used 5' GAC TGG TTC TGT GGT GG 3' represents the complementary sequence of bases 688–672 of RSV G gene and the RT-PCR was applied according to Alansari and Potgieter (1993); Vilcek *et al.* (1994).

Primers for BVD:

Oligonucleotide primers from 5'UTR of virus genome were 5'GAT GGC TTA AGC CCT GAG TA-3' and 5'GCT GTA TTC GTA ACA GTC GG 3' used for RT reaction and subsequent PCR amplification as described Sasaki *et al.* (1996).

C- Electron microscopy: Some of the harvested positive samples suspension was used for electron microscopy at Electron Microscope unite, Vaccines and Sera Institute (VACSERA), according to Madbouly *et al.* (2005).

Bacteriological Assay:

The swabs were plated onto 5% sheep blood and MacConkey's agar for 24 hrs at 37°C under aerobic conditions and the growth of the different bacterial species was recorded on a semi-quantitative scale: pure culture, a few bacteria in mixed culture and no growth. The plates were then examined for growth, colony morphology, colour and odour. Bacterial identification was conducted according to the standard protocol described in Cowan Steel's manual (1974) while biochemical testing (API 20NE, BioMérieux) was used to identify *P. multocida* and/or *M. haemolytica* (Eidam *et al.*, 2013).

Statistical assay:

Data were computed and statistically analyzed using Chi square analysis (Sndecor and Cochran, 1980).

3. Results and Discussions

Epidemiology and Clinical Signs:

Out of 227 examined buffalo-cows, 92 (40.53%) animals were positive for BRDC. Infected animals showed clear clinical signs of the disease only in 59 animals (25.99%) while 66.96% of these animals did not show any signs (Table 1). A highly significant ($P < 0.01$) percent (16.74%) of animals showed signs of BRDC revealed more than one sign, while fever, cough, pneumonia, nasal and ocular discharges were the most observed clinical signs in BRDC infected animals (Table 2).

Virus identification by Dot ELISA:

Dot ELISA revealed that 14.54% of serum and nasal swabs samples from apparently healthy Buffalo-cows (33 out of 227) were positive for BRDC. However, 100% of the samples collected from animals showing the disease symptoms contained BRDC (Fig. 1).

Monoplex PCR and RT-PCR

PCR assay indicated that there is bands belonged to viral protein encoding gene e.g. for BHV-1 the band was at 629 bp which belonged to glycoprotein gI sequence which come in agreement with Van Engelenburg *et al.* (1993), for PI-3 the band was at 400 bp representing coding of F gene and similar result was given by Lyon *et al.* (1997); Zhu *et al.* (2011), for BRSV the band was at 426 bp belonged to G gene as in Alansari and Potgieter (1993); Vilcek *et al.* (1994) and for BVD the band was at 160 bp corresponding to 5'UTR of virus genome which come in agreement with the findings of Sasaki *et al.* (1996) (Fig. 2).

Electron microscopy:

Examination of sodium phosphotungstate stained drops of several samples by electron microscopy, showed BHV-1 which is approximately 125 nm in diameter. Surrounding the capsid is a layer of globular material, known as the tegument, which is enclosed by a typical lipoprotein envelope with numerous small glycoprotein spikes which matches the results of Liang *et al.* (1996) ; Ravishankar *et al.* (2012). PI-3 is pleomorphic in shape (spherical and filamentous), 150- 200 nm in diameter. The virions have envelope covering it with large peplomers also BRSV resembles that of PI-3 because both belonged to the same family which come in agreement with Horwood *et al.* (2008) ; Zhu *et al.* (2011). Moreover, BVD is 50 nm spherical enveloped virions this result match the findings of Givens *et al.* (2003) (Fig.3).

Moreover, table (3) reveals that for all the investigated viruses, RT-PCR (Fig. 3.1 – 3.4) detected significantly ($P< 0.01$) higher incidence as compared to Dot ELISA (Fig. 1A- 1D) in both BRSV and BVDV samples and significant ($P<0.05$) incidence in BHV-1 and PI-3 samples this result attributed to the high sensitivity of PCR test compared to any conventional diagnostic test and these results comes in agreement with Horwood and Mahony (2010); Thonur *et al.* (2012).

Bacteriological assay:

Bacteria were cultivated from 227 nasal swabs, with *Pasteurella* (n = 34) and *Mannheimia* (n = 24) (Table 4). Colonies of *M. haemolytica* were small and grey, with a narrow zone of haemolysis after 18–24 hrs of incubation. In the API system, all the isolates were indole negative and fermented arabinose. *P. multocida* was found in single infection

(n = 7) and in double infection (n = 18) and in triple infection (n=9). On the other hand,, *M. haemolytica* was found in single infection (n = 5) was isolated from animals suffering from double infection (n=17) and in triple infection (n= 2) (Table 5).

The most observed result was the frequent association between BVD with *M. haemolytica* (n = 7) followed by PI-3 with *P. multocida* (n = 6), BHV-1 with *P. multocida* (n=5), BHV-1 with *M. haemolytica* (n= 5), BRSV with PI-3 (n = 5) and the BRSV-PI-3 (n = 5). In the case of triple infections (9/92), the interaction between BRSV- PI-3 and *P. multocida* was observed in four cases, then the association between BHV-1 and PI-3 *P. multocida* (n=2), while association of BRSV-*P. multocida* and *M. haemolytica* as well as PI-3- *P. multocida*- *M. haemolytica* and BRSV- BVD - *P. multocida* was observed once each (Table 5).These results comes in agreement with Fulton *et al.* (2009) where association between viral and bacterial pathogen was observed forming BRDC.

The current investigation indicates that BRDC in Egyptian buffalo-cows shows fever, cough, pneumonia, nasal and ocular discharges. It also suggests that the etiopathogenesis complex of acute pneumonia and resultant fatality could be a result of pathogenic interaction between viruses (BHV-1, BRSV, PI-3, BVD) and bacteria (*P. multocida* and/or *M. haemolytica*). The study also does not discard the participation of other potential pathogens, such as *Mycoplasma sp.* or Bovine *coronavirus*, reported by Hung *et al.* (1991) and Decaro *et al.* (2008), in the development of similar lung infections in buffalo-cows. Moreover, the current study investigated suitable and available diagnostic tests for BRDC.

Table 1: Overall incidence of BRDC in buffalo-cow:

Number of examined animals	Number of BRDC positive animals			
	Number of animals showing signs of the disease		Number of animals did not show signs of the disease	
	Number	Percentage	Number	Percentage
227	59	25.99%	33	14.54%

Table 2: Clinical signs of BRDC in buffalo-cow:

Clinical signs	BRDC positive animals	
	Number	Percentage
Fever	48	21.15%
Cough	49	21.59%
Nasal and ocular discharges	55	24.23%
Pneumonia	52	22.91%
More than one clinical signs from the above mentioned	38	16.74%**
λ^2	2.83**	

**$P < 0.01$ and *$P < 0.05$.

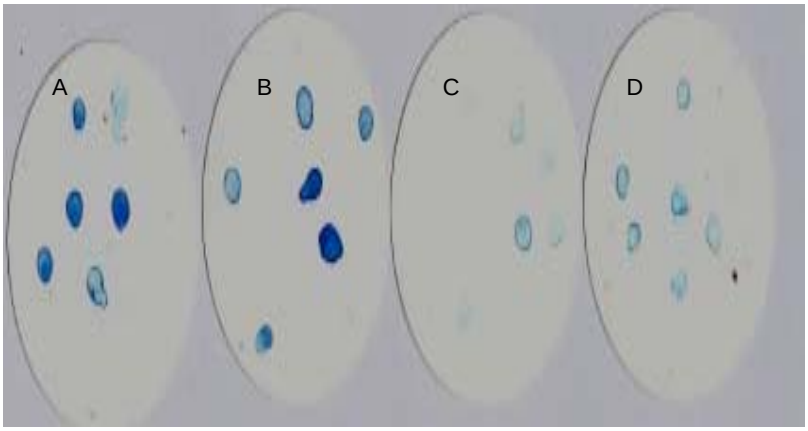

Fig1: Dot ELISA show positive and negative samples with different intense color. A: Tested samples against BHV-1. B: Tested against PI-3. C: Tested against BRSV. D: Tested against BVD.

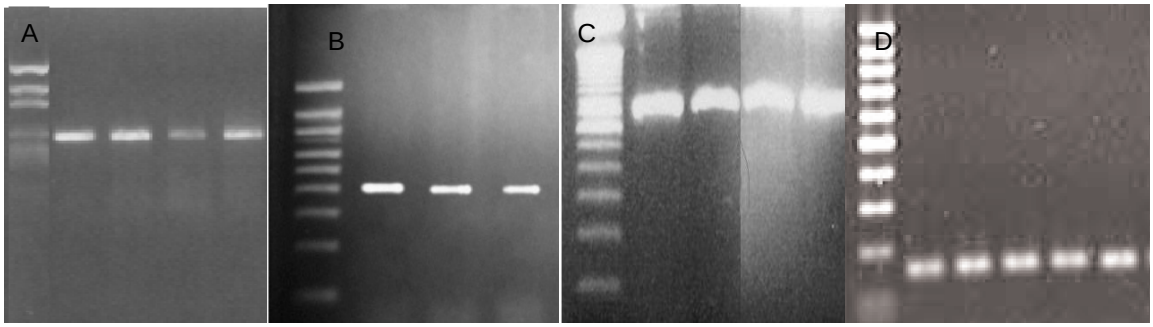

Fig. 2 agrose gel electrophoresis A: PCR of g1 gene of BHV-1 where the band was at 629bp. B: RT-PCR of PI-3. There was a band at 400bp of F gene. C: RT-PCR for G gene of BRSV where the band was at 426 bp. D: RT-PCR for 5'UTR of virus genome and the ban was at 160 bp. The ladder used was a 100 bp DNA ladder (QIAGEN) (as a molecular marker).

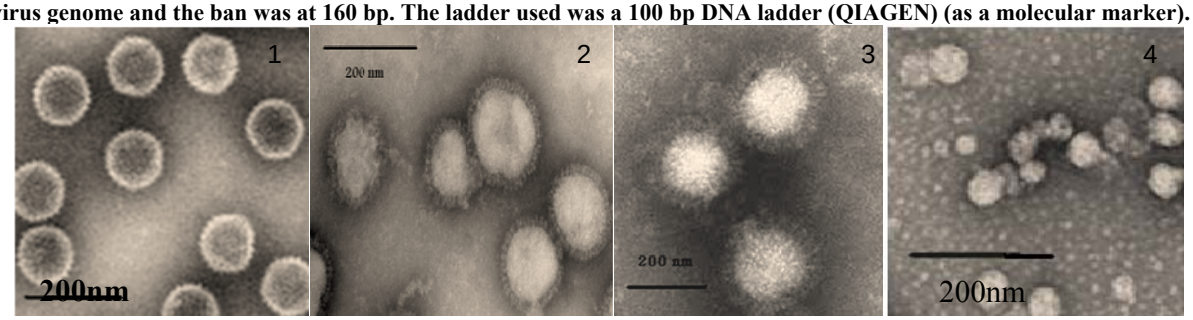

Fig. 3: Negative stained electron microscopy showing: 1: 125nm envelop virions which contain tegument with small spikes on it. 2: PI-3 spherical and filamentous virions with peplomers visible in edge. 3: BRSV is approximately 130 nm pleomorphic virions with large spikes. 4: small spherical virus app. 50 nm identified as BVDV.

Table 3: Detection of viral causative agents of BRDC using dot ELISA and PCR%:

Method	BHV-1	PI-3	BRSV	BVD
Dot-ELISA	22 ±1.76*	17±1.36**	11±0.88**	25±2
PCR	29±2.32	19±1.52*	15±1.2**	29±2.32
λ^2	5.38*	4.33*	0.41**	1.08**

**P < 0.01 and *P < 0.05

Table 4: Bacteria isolated from nasal swabs

Numbers of samples	Positive cultures for *P. multocida*	Positives cultures of *M. haemolytica*
227	**29**	**24**
	12.78%	**10.57%**

Table5: Association between viral and bacterial pathogens:

Number of pathogenic agents	Pathogenic agent		Positive results	
Single infection	Viral agent	BHV-1	17±0.4	7.49%
		PI-3	1±0.3	0.44%
		BRSV	1±0.3	0.44%
		BVD	12±0.07**	5.29%
	Bacterial agent	*P. multocida*	7±0.009**	3.08%
		M. haemolytica	5±0.06**	2.20%
Double infection	BRSV with *P. multocida*		3±0.1	1.32%
	BHV-1 with *M. haemolytica*		5±0.06**	2.20%
	BHV-1 with *M. haemolytica*		5±0.06**	2.20%
	BVD with *M. haemolytica*		7±0.009**	3.08%
	PI-3 with *P. multocida*		6±0.03**	2.64%
	BRSV with PI-3		5±0.06	2.20%
	BVD with *P. multocida*		4±0.09**	1.76%
	BVD with BHV-1		5±0.06**	2.20%
Triple infection	BRSV- PI-3 and *P. multocida*		4±0.09**	1.76%
	BRSV-*P. multocida* and *M. haemolytica*		1±0.3**	0.44%
	BHV-1 and PI-3 *P. multocida*		2±0.2**	0.88%
	PI-3- *P. multocida* and *M. haemolytica*		1±0.3**	0.44%
	BRSV- BVD and *P. multocida*		1±0.3**	0.44%
λ^2	0.15**			

**P < 0.01

Corresponding Author:
Dr. Kawther S. Zaher,
Microbiology and Immunology Department, National Research Center (NRC), Dokki, Cairo, Egypt.
Email: zaherkus@yahoo.com

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3/19/2014