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Preparation and Evaluation of Fluorescein Isothiocyanate-Conjugated Polyclonal Antibodies for Rapid Detection of Duck Viral Enteritis (Duck Plague)

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Abstract

The main goal of the current study was to prepare and evaluate the suitability of fluorescein-conjugated anti-Duck enteritis virus polyclonal antibodies for routine diagnosis and research purposes. A total of 22 animals (10 rabbits, 10 guinea pigs and 2 goats) were immunized subcutaneously with Live attenuated DEV vaccine (Jansen strain) containing 106 EID50/ml. Rabbits and guinea pigs received 0.5 ml per dose, meanwhile goats received 1ml. A total of five doses with 7 day intervals were applied and the last dose was simultaneously injected with Montanide™ ISA 206 oil adjuvant. Five weeks post-immunization, DEV-specific SN antibody titers were significantly increased and reached to the peak level with a mean titer of 32, 128 and 256 in guinea pig, rabbit and goat sera, respectively. The globulin fraction of the prepared antisera was successfully precipitated with ammonium sulphate and then fluorescein-conjugated. The application of direct fluorescent antibody technique test was carried out on infected Vero cells with DEV on cover slips, revealed that positive reactions were obtained with guinea pig, rabbit and goat conjugates up to a dilution of 10-4, 10-5 and 10-6respectively. These findings clearly indicate that fluorescein-conjugated polyclonal antibodies developed in the present study can be used for accurate and precise diagnosis of DEV.

1. INTRODUCTION

Duck viral enteritis, also known as duck plague, is an acute contagious herpes virus infection of ducks, geese, swans and other water fowl within the order Anseriformes (Davison et al., 1993). The virus was classified as a member of family herpesviridae, subfamily Alphaherpesvirinae, Mardivirus genus (ICTV, 2014). In Egypt, the disease was first reported in large flock of white Pekin ducks in Bahtim Province (Sabry et al., 1986). It is characterized by causing great economic losses with mortality rate can reach up to 16% in breeders and 40% in broilers as well as drop in egg production ranged from 0.5-99.5% (Sultan, 1990; Kheir El Dine et al., 1992). Under natural conditions, susceptible birds are usually infected through close contact to diseased birds (Kaleta et al., 2007). Migratory and domestic waterfowl may spread the infection from one another (Kathryn et al., 2001; Wang et al., 2013). The disease has been reported in birds ranging from 7 days of age to mature breeders. The incubation period varies from 3 to 7 days, and death usually occurs 1-5 days after the onset of clinical signs and is often more severe in susceptible adult breeder ducks. The range of signs include sudden deaths, photophobia associated with partially closed and pasted eye-lids, loss of appetite, ataxia, and nasal discharge. In ducklings 2–7 weeks of age, losses may be lower than in older birds and the signs include dehydration, loss of weight, conjunctivitis and serous

ocular discharge, a blue colouration of the beaks and blood-stained vents (OIE, 2012).Duck enteritis virus (DEV) can be identified by virus neutralization test (Wu et al., 2011), passive haemagglutination test (Das et al., 2009), by inoculation into 11-13 days old duck embryo through chorioallantoic membrane route (Hanaa et al., 2013), by propagation in duck embryo fibroblast cell culture (Gao et al., 2014) and finally by molecular detection using polymerase chain reaction (PCR) (Hansen et al., 2000).Fluorescent antibody technique (FAT)has been used for studying tissue localization of duck plague virus (Tantaswasdi et al., 1988). Immunohistochemistry examination conducted on specimens (spleen, thymus, liver and duodenum) collected from artificially infected ducks indicated thatUL24 gene encoding protein of DEV could be detected in almost all the tissues 24 hours post infection, meanwhile, could be detected by FAT and PCR detection methods 12 hours post infection (Jia, 2008). The present study aimed to prepare and evaluate the applicability of fluorescein-conjugated DEV antisera for laboratory diagnosis of DEV using direct FAT.

2. MATERIALS AND METHODS

2.1.Virus strain and vaccine

Vero cell culture adapted DEV (Yasumara and Kawatika, 1963) with a titer of 6log10 TCID50/ml was was kindly supplied by Veterinary Serum and Vaccine Research Institute (VSVRI), Abbassia, Cairo. The virus was used for titration of the prepared hyperimmune sera using serum neutralization test (SNT).

Live attenuated DEV vaccine (Jansen strain) was kindly supplied by VSVRI was used for preparation of anti-DEV hyperimmune sera in different animal species.

2.2.Animals

A total of ten white male rabbits (Boskat breed) weighing about 3 kg, ten male guinea pigs weighing about 800 g and two male goats (5-8 months) were used for preparation of hyperimmune sera. In addition, two animals from each species were used as a negative control. All animals were purchased from a local market, and then housed under hygienic conditions in battery cages (rabbits and guinea pigs) and pens (goats). Feed and water were provided for ad libitum consumption.

2.3.Raising of anti-DEV hyperimmune sera

After acclimatization and deworming, animals were immunized subcutaneously with live attenuated DEV vaccine (Jansen strain) containing 106 EID50/ml. Rabbits and guinea pigs were injected subcutaneously in the mid portion of the neck with 0.5 ml, whereas goats received 1ml by the same route. For all animals, five doses were injected with 7 day intervals. The last injection was accompanied with Montanide™ ISA 206 oil adjuvant(Abd El-Waneis and Khodeir 2004). Sera were obtained from all animals at weekly intervals post immunization to follow up the levels of induced antibodies using SNT.

2.4.Evaluation of the prepared anti-DEV hyperimmune sera using SNT

DEV-specific SN antibody titers were estimated in the prepared antisera according to the method described by Bass et al., (1982). Briefly, the serum samples were initially incubatedin a water bath at 56°C for 30 minutes. About, 25µl of serial two-fold dilutionsof the serum samples were mixed with 25µl of DEV suspension (containing 100 TCID50/ml) in a 96-well tissue culture plates. Five wells were allocated for each serum dilution. The virus-serum mixtureswere incubated at 37°C for 60 minutes with frequent agitation. To each well, 150µl of African green monkey kidney (Vero) cell suspension (containing approximately 15000 cells in Eagle's MEM supplemented with 10 % newborn calf serum) were pipetted into each well, after which the plates were incubated again at 37°C and examined daily up to 7 days for the appearance of cytopathic effect (CPE).Neutralizing antibody titers were expressed as the reciprocal of the highest serum dilution that completely inhibited virus-induced CPE of 100 TCID50 virus (Wu et al., 2004).

2.5.Measurement of serum total protein, albumin and globulins

Serum total protein levels (gm/dl) were assayed by using the biuret colorimetric method (Young and Friedman, 2001) and serum albumin was determined according to Burtis et al., (1999). Globulin was calculated by subtracting albumin concentrations from the total protein levels (Coles, 1986). The assays were performed using Reactivos GPL kits (Barcelona, Espana, Spain).

2.6.Precipitation of the prepared immunoglobulins.

The globulin fraction was precipitated according to the method described by Narin and Marrck (1964) and then adjusted to be 20mg/ml using phosphate buffer saline.

2.7.Preparation of fluorescein-conjugated immunoglobulins

The conjugation of immunoglobulins with fluorescein isothiocyanate (FITC) was carried out according to the method of Nairn (1969). All chemicals used were supplied by Merck, Darmstadt for Microscopy (M.Gew.389.39).The residual untreated FITC and labeled protein were removed according to Corbel(1973).

2.8.Evaluation of the conjugated immunoglobulins by FAT

Direct FAT was carried out on infected Vero cells with DEV to evaluate the prepared DEV-antisera conjugated with fluorescein isothiocyanate. The technique was carried out according to Soliman et al (1989). Briefly, fixed coverslip cell cultures (DEV-infected and non-infected control) were washed briefly with three changes of phosphate buffered saline (PBS). After air drying, the cells were covered with one drop of DEV anti-serum conjugated with FITC and incubated for 60 minutes at 37oC in humidified CO2 incubator. They were subsequently rinsed three times with PBS (pH 7.4), then mounted in phosphate buffered glycerin (1:10) and finally observed under the fluorescence microscope (VEB Carl Zeiss JENA-DDR) with 500x magnification.

3. RESULTS AND DISCUSSION

Among duck diseases, duck viral enteritis is the most feared disease (Hanan et al. 2014). Accurate and rapid detection of the virus is crucial for successful controlling the disease. FAT based on rabbit anti-DEV polyclonal serum and an anti-rabbit FITC conjugate was proved to be a rapid test for the detection of DEV in impression smears prepared from tissues taken from experimentally infected ducks. Results of FAT can be available in 2 to 3 hours of samples being received at the laboratory (Morrissy, 2004).Polyclonal antibodies (PAbs) are relatively easy and inexpensive to prepare in a relatively short time frame (e.g., 3–4 months) and are also more stable over a broad pH range and salt concentrations. Moreover, polyclonal sera are a composite of antibodies with unique specificities and frequently have better specificity than monoclonal antibodies because they are produced by a large number of B cell clones each generating antibodies to a specific epitope (Lipman et al., 2005; Jimenez-Lopez and Hernandez-Soriano, 2012).The present study aimed to provide free and FITC-conjugated anti-DEV polyclonal antibodies to be used as local reliable reagents for rapidand accurate diagnosis of DEV using direct FAT. PAbs raised to the same antigen using multiple animals will differ among immunized animals, and their quantity obtained is limited by the size of the animal and its lifespan (Lipman et al., 2005). In the present study a total of 22 animals (10 rabbits, 10 guinea pigs and 2 goats) were subcutaneously hyperimmunized with DEV. At weekly intervals starting on the day of first immunization, DEV-specific antibody titers were estimated in the collected sera by SNT. The geometric mean SN antibody titers against DEV were significantly increased in the sera of all immunized animals until the 5th week post immunization (Table 1). In comparison with rabbits and guinea pigs, goats had a considerably higher antibody titer. Our findings are also supported by Alkhalefa et al., (2009), who found that the mean SN antibody titers raised against Newcastle disease virus in rabbits and goats were 7 and 9 log2, respectively. Meanwhile, mean SN antibody titers raised against infectious bronchitis virus and infectious bursal disease virus in rabbits and goats were 8 and 7 log2, respectively.Generally, sera of immunized animals had a significantly increased total protein and globulin levels in comparison with control group (Table 2). In agreement with (Caporale et al., 2009), higher immunoglobulins concentrations were obtained by ammonium sulphate precipitation and then adjusted to be 20mg/ml as recommended by Perrin (1996). In order to evaluate applicability of the conjugated immunoglobulins for virus diagnosis, DEV-infected and non-infected control vero cells fixed on coverslips were tested by direct FAT using ten-fold serial dilutions of the conjugated immunoglobulins. Results revealed that guinea pig, rabbit and goat anti-DEV Fluorescin-conjugated immunoglobulins showed clear strong positive reactions appear as apple green dots (Figure 1A) up to dilution of 1/100, 1/100 and 1/1000, respectively. Moderate positive reaction (Figure 1B) was obtained using 1/10000 dilution of rabbit conjugate while such degree was obtained with a dilution of 1/100000 using goat conjugate. Weak positive reaction (Figure 1C) was noticed with a dilution of 1/10000, 1/100000 and 1/1000000 of guinea pig, rabbit and goat conjugates, respectively. Negative reactions (Figure 1D) were obtained with further dilutions of the three conjugates as well as unconjugated sera. In a previous study (Fakhry et al., 2013), Fluorescein-conjugated antisera obtained from immunized guinea pigs, rabbits and goats with FMDV SAT2 strain showed clear positive reaction up to a dilution of 1/8000, 1/10000, 1/10000, respectively. In conclusion, the locally prepared fluorescein-conjugated DEV polyclonal antibodies either in goats

or rabbits could be successfully used for accurate and precise diagnosis of DEV by direct FAT.

Table (1): Mean DEV-SN antibody titers in the sera of immunized animals.

Antisera	Mean antibody titers					
	0 day	7th day	14th day	21st day	28th day	35th day
Rabbits	0	2	8	32	64	128
Guinea pigs	0	≤2	4	16	32	32
Goats	0	4	16	32	128	256

Table (2): Levels of total proteins, albumin and globulin in sera of immunized and control animals.

	Rabbits		Guinea pigs		Goats	
	Immunized	Control	Immunized	Control	Immunized	Control
Total number	10	2	10	12	2	2
Total proteins (gm/dl)	5.45±0.11	4.6±0.21	5.35±0.13	4.8±0.23	5.80±0.11	5.14±0.22
Albumin (gm/dl)	1.90±0.22	2.0±0.15	1.19±15	2.5±0.10	1.51±0.32	2.07±0.18
Globulin (gm/dl)	3.55±0.12	2.6±0.30	4.16±10	2.3±11	4.29±0.10	3.07±0.11

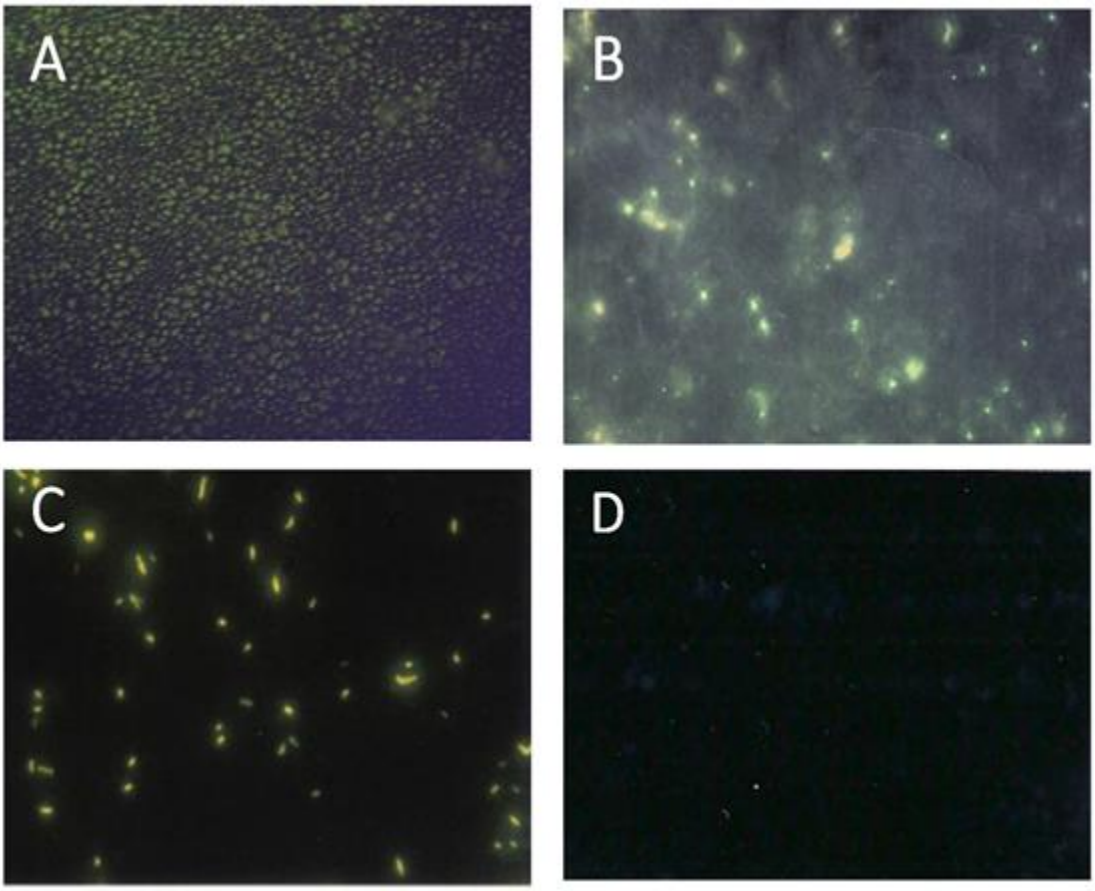

Figure (1): Results of DEV identification in tissues of experimentally infected rabbits using direct FAT based on ten-fold serial dilutions of fluorescein conjugates; strong positive reaction (A), moderate positive reaction (B), weak positive reaction (C) and negative reaction (D).

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