Journal of Virological Methods 251 (2018) 1–6

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Intracellular fixation buffer inactivates Newcastle disease virus in chicken allantoic fluid, macrophages and splenocytes

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ARTICLE INFO

Keywords:
Newcastle disease virus
Virus inactivation
Formaldehyde
Flow cytometry
HD11
CD8
CD4
Cellular fixation

ABSTRACT

Inactivation of Newcastle disease virus (NDV) has been routinely achieved with heat, β-propiolactone, binary ethylenimine, ultraviolet light and formalin. However, these strategies have not been tested for cell surface ligand or receptor phenotype in viral-infected chicken immune cells. To study the capacity of fixation buffers to preserve surface markers while inactivating NDV, a primary splenocyte culture was infected with NDV and incubated with a commercial intracellular fixation buffer (ICB), formulated with 4% formaldehyde. Splenocytes were fixed with a 1:2 dilution of ICB in phosphate buffered saline (PBS) for 45 min at 23 °C or 4 °C and inactivation of NDV was tested in addition to recognition of antigens by antibodies in fixed and non-fixed splenocytes via flow cytometric analysis. The binding and percentage of splenic CD4+ and CD8+ cells were not affected. In addition, NDV titers as high as $10^{9.5}$ and $10^{7.6}$ EID_{50} in allantoic fluid (AF) and macrophages, respectively, were successfully inactivated after 45 min at 23 °C and 4 °C, confirming the ICB's effectiveness in inactivating high concentrations of NDV. In conclusion, high concentrations of NDV in AF, chicken splenocytes, and macrophages can be inactivated using ICB. Additionally, this method did not compromise cell phenotyping of enriched chicken splenocytes.

1. Introduction

Newcastle disease virus (NDV) can infect over 270 avian and non-avian species (Miller and Koch, 2013). In poultry, virulent strains can lead to 100% mortality and morbidity of the flock (Alexander, 2003). Velogenic or virulent forms of NDV (vNDV) are considered biological select agents, as they can pose a severe threat to poultry health and safety. Since current biosecurity measures and vaccination protocols have been unable to prevent outbreaks worldwide, especially in developing countries, it is critical to ensure the safe handling of NDV in the laboratory setting (Nath et al., 2016; Sun et al., 2013; Shabbir et al., 2013; Kammon et al., 2015; Jaganathan et al., 2015; Ganar et al., 2014). While research efforts have focused on developing improved vaccines against NDV (Cardenas-Garcia et al., 2015; Miller et al., 2009; Kim et al., 2013; Miller et al., 2013), mechanistic research in to characterizing the avian immune response to viral pathogens is still needed to optimize vaccines that are highly effective. Specifically, continued studies focusing on the role of and type of humoral and cellular immune response associated with NDV infection at multiple stages of exposure.

Experiments to specifically study the innate and adaptive responses to NDV require timed infection exposures with the activated and inactivated virus. To conduct vaccination experiments that allow evaluation of the bird's immune system, samples need to be transported from a biosafety level (BSL) 2facility to a BSL1facility to perform a number of immune-based assays, such as flow cytometric analysis. It is therefore crucial for the virus to be completely inactivated. In the present study, we evaluated a fixation procedure that would eliminate the risk of transferring live NDV to lower biosafety levels and minimize release of the virus into the environment, while allowing cellular preservation for select immune studies.

Inactivation of NDV can be achieved using heat, β-propiolactone (BPL), binary ethylenimine, formalin or ultraviolet (UV) light (King,

Abbreviations: NDV, Newcastle disease virus; vNDV, velogenic NDV; DPI, days post infection; BPL, β-propiolactone; UV, ultraviolet; ICB, intracellular fixation buffer; AF, allantoic fluid; SPF, specific-pathogen-free; MDT, mean death time; ICPI, intracerebral pathogenicity index; LS, LaSota; LS-RFP, LS with the red fluorescent protein; ECE, embryonated chicken eggs; EID_{50}, embryo infectious dose 50%; HA, hemagglutination assay; MOI, multiplicity of infection

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http://dx.doi.org/10.1016/j.jviromet.2017.09.025
Received 18 June 2017; Received in revised form 16 September 2017; Accepted 19 September 2017
Available online 29 September 2017
0166-0934/ Published by Elsevier B.V.

1991; Swayne and Beck, 2004; Qayyum et al., 1999; Sutton et al., 2013). However, currently available methods for NDV inactivation have not being designed or tested for use in immunological studies and some may compromise the structure of the plasma membrane of the host cells, while others have not been tested for their efficacy against high viral titers. In addition, the noted shortcomings of these inactivation methods include inactivation inconsistency, inactivation of low NDV titers, incomplete confirmation of inactivation through several passages in eggs that extend at least 5 days per passage and lack of testing with splenic lymphocytes and flow cytometric analysis. Sutton et al. (2013), successfully inactivated NDV using UVB light however, other studies have been unable to inactivate NDV using UVC light demonstrating that further research needs is needed to ascertain whether this method can reliably be used or even proposed as an effective protocol (Qayyum et al., 1999; Sutton et al., 2013).

Qayyum et al. (1999) achieved successful inactivation of NDV using heat for 45 min, a pH of 1 and 13 at 6 h, 0.48% formalin in 30 min, and several concentrations of disinfectants such as Phenol crystals, iosan and bromosept at 15 and 30 min (Qayyum et al., 1999). Nevertheless, they were unable to successfully inactivate the virus with UV light. However, their evaluation was not as sensitive as the one performed in the present study as only 2 HA units were used for inactivation, only one passage of eggs was performed to verify viral inactivation and the eggs were only incubated for 48 h.

Previous studies by King (1991) showed that inactivation of NDV was possible using 0.04 and 0.1% formalin for 16 h at 37 °C, 0.025% and higher concentrations of BPL for 18 h at 4 °C, and heat (60 °C) in less than 30 min (King, 1991). In comparison, the current protocol in this study used 4% formalin. This increase in concentration greatly reduced the inactivation time from 16 h to 45 min, allowing for same day analysis of samples, more efficient use of personnel time and faster data acquisition. King (1991) was unable to inactivate high concentrations of NDV in 120 min at 56 °C, but achieved inactivation in less than 30 min at 60 °C. Swayne et al. also achieved inactivation of NDV using high temperatures ranging from 55 °C and 63 °C (Swayne and Beck, 2004). Although very successful in inactivating NDV, heat inactivation is not suitable for immune phenotyping via flow cytometry. This can lead to protein denaturing, which negatively impacts antibody cell surface protein recognition binding during the immune phenotyping procedure. These conformational changes can be prevented at least short term with formaldehyde fixation (Mason and O'Leary, 1991).

The aim of the present study was to test the effectiveness of a commercial intracellular fixation buffer (ICB), which is based on a 4% formaldehyde solution, as an appropriate inactivation procedure for NDV. Formalin is often used as a cellular fixative for flow cytometric analysis and as a tissue fixative for histopathology (Cardenas-Garcia et al., 2016). We hypothesized that ICB can inactivate high concentrations of NDV without compromising phenotype identification using flow cytometry. For this purpose, we modified a fixative protocol normally used for fixation of lymphocytes for flow cytometric analysis using ICB (Cardenas-Garcia et al., 2016). This procedure was adapted to inactivate NDV from allantoic fluid (AF), infected chicken macrophage HD11 cells and infected splenocytes isolated from specific pathogen free (SPF) chickens.

2. Materials

2.1. Viruses

All viruses used were lentogenic NDV strains, or strains of high pathogenicity that have been attenuated by a change in the fusion protein cleavage site. Recombinant ZJ1-L is an attenuated version of ZJ1 (Goose/China/ZJ1/2000; GB AF431744.3) that was previously generated in our laboratory through reverse genetics. Recombinant ZJ1-L has a mean death time (MDT) of over 175 h and an intracerebral

Table 1
Effect of 23 °C and 4 °C fixation on inactivation of three lentogenic NDV strains in AF.

		Incubated at RT		Incubated at 4 °C	
		Total Dead	HA +	Total Dead	HA+
Negative Controls	BHI	0	0/3	0	0/3
	BHI + ICB	0	0/3	0	0/3
100 µl virus 100 µl ICB	LS + ICB	0	0/5	0	0/5
	rZJ1*L + ICB	0	0/5	0	0/5
	LS-RFP + ICB	1*	0/5	0	0/5
50 µl virus 150 µl ICB	LS + ICB	0	0/5	0	0/5
	rZJ1*L + ICB	0	0/5	0	0/5
	LS-RFP + ICB	0	0/5	0	0/5
150 µl virus 50 µl ICB	LS + ICB	0	0/5	0	0/5
	rZJ1*L + ICB	0	**3/5**	0	1/5
	LS-RFP + ICB	0	**1/5**	0	0/5
Virus alone	LS	3	3/3	3	3/3
	rZJ1*L	3	3/3	3	3/3
	LS-RFP	2	3/3	2	3/3
Passage 2					
100 µl virus 100 µl ICB	LS + ICB		0/5	1	0/5
	rZJ1*L + ICB		0/5		0/5
	LS-RFP + ICB	2	0/5	1	0/5
50 µl virus 150 µl ICB	LS + ICB	1	0/5		0/5
	rZJ1*L + ICB		0/5	1	0/5
	LS-RFP + ICB		0/5		0/5
	*LS-RFP + ICB		0/5		

* The mortality of this egg was unexpected; therefore this individual egg was passed onto another 5 eggs.

Table 2
Effect of 4 °C fixation on inactivation of LaSota (LS) strain-infected HD11 cells.

Passage 1		Eggs Inoculated	Total Mortality	HA+
Media Controls	Not fixed or lysed	5	0	0/5
	Lysed Cells	5	0	0/5
	Fixed and Lysed cells	5	0	0/5
	Fixed cells	5	0	0/5
Virus Inoculated Cells	Fixed, Lysed cells	15	0	0/15
	Fixed, Not lysed	15	0	0/15
	Lysed, Non-Fixed cells	Calculated titer of virus was $10^{7.6}$ EID_{50}/mL		
	Non- Lysed, Non-Fixed cells	Calculated titer of virus was $10^{7.5}$ EID_{50}/mL		
Passage 2				
Virus Inoculated Cells	Fixed and Lysed cells	**15**	1	0/15
	Fixed, Not lysed	**15**	0	0/15

Table 3
Effect of RT or 4 °C fixation on inactivation of LaSota (LS) strain-infected splenocytes isolated from adult SPF White Leghorns.

Passage 1	Eggs Inoculated	Total Mortality	HA+
RT Media Controls	5	0	0/5
4 °C Media Control	5	0	0/5
RT Fixed	5	0	0/5
Passage 2			
LS Fixed Cells 4 °C	15	0	0/15
LS Fixed Cells RT	15	0	0/15