

Using immunoglobulin Y as an alternative antibody for the detection of hepatitis A virus in frozen liver sections

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An increasing amount of research has been conducted on immunoglobulin Y (IgY) because the use of IgY offers several advantages with respect to diagnostic testing, including its easy accessibility, low cost and translatability to large-scale production, in addition to the fact that it can be ethically produced. In a previous work, immunoglobulin was produced and purified from egg yolks (IgY) reactive to hepatitis A virus (HAV) antigens. In the present work, this anti-HAV-specific IgY was used in an indirect immunofluorescence assay to detect viral antigens in liver biopsies that were obtained from experimentally infected cynomolgus monkeys. Fields that were positive for HAV antigen were detected in liver sections using confocal microscopy. In conclusion, egg yolks from immunised hens may be a reliable source for antibody production, which can be employed for immunological studies.

Key words: immunoglobulin Y - hepatitis A virus - indirect immunofluorescence

Immunoglobulin Y (IgY) is the major antibody produced by chickens (*Gallus gallus domesticus*). It is continually synthesised at a large scale, secreted into the blood and transferred to the egg yolk, where it accumulates (Warr et al. 1995). To obtain specific IgY antibodies against an antigen of interest, egg-laying hens are immunised with the antigen; following this, antibodies are purified from their egg yolks. This noninvasive method of obtaining antibodies is one of the advantages of using IgY in virological immunodiagnoses. Additional advantages of using IgY include the generation of a better immune responsiveness to mammalian antigens due to the phylogenetic distance between hens and mammals, the ability to generate IgY antibodies that have high avidity and with Fc regions that do not bind to Fc receptors in mammals, the fact that IgY does not activate the complement system and its lack of interaction with rheumatoid factor (Schade et al. 2005). Compared to immunoglobulin G purification, IgY purification is an easy, fast and low-cost isolation process that produces a high quantity of specific antibody. Therefore, IgY can be used in various immunological assays and may offer better results than traditionally used mammalian antibodies (Kricka 1999).

Infection with hepatitis A virus (HAV) occurs via the faecal-oral route and is the most common cause of acute viral hepatitis in Brazil. The incidence rate of

HAV is closely correlated to socio-economic conditions, hygiene status and access to safe drinking water (Franco et al. 2012). HAV infection spreads easily, either by personal contact or by the ingestion of contaminated food and water (Vital et al. 2006). The infection is generally self-limiting and can produce effects that range from an absence of symptoms to death from fulminant hepatitis (Nainan et al. 2006). IgY has been widely used in the diagnosis of infectious diseases, in methods such as immunofluorescence, immunohistochemistry, immunoenzymatic assay (ELISA) and western blotting (WB) (Tini et al. 2002, Young et al. 2007). The detection of HAV in liver sections is a useful tool for identifying acute or fulminant hepatitis cases in which IgM is undetectable in blood samples using commercial immunoassays and in cases where the serum viral load is either too low or unable to be detected using molecular RNA detection techniques (Shimizu et al. 1982, Ferreira et al. 2008). Rezende et al. (2003) concluded that low viral load was the primary factor associated with acute liver failure, presumably owing to a strong host immune response.

In a previous study, we demonstrated the use of anti-HAV IgY as a capture antibody in an “in-house” ELISA (da Silva et al. 2012). Here, we suggest the effectiveness of using IgY as a diagnostic measure of HAV in frozen liver sections from monkeys via indirect immunofluorescence (IIF).

The IgY used in this study was obtained during previous work performed by our group (de Paula et al. 2011), in which hens were immunised with HAV antigens combined with adjuvants (incomplete Freund's adjuvant and CpG-oligodeoxynucleotides, CpG-ODN). Eggs were collected and the immunoglobulin was purified by precipitation using the polyethylene glycol (PEG) method described by Polson et al. (1985). Next, the antibody was characterised by sodium dodecyl sulfate polyacrylamide

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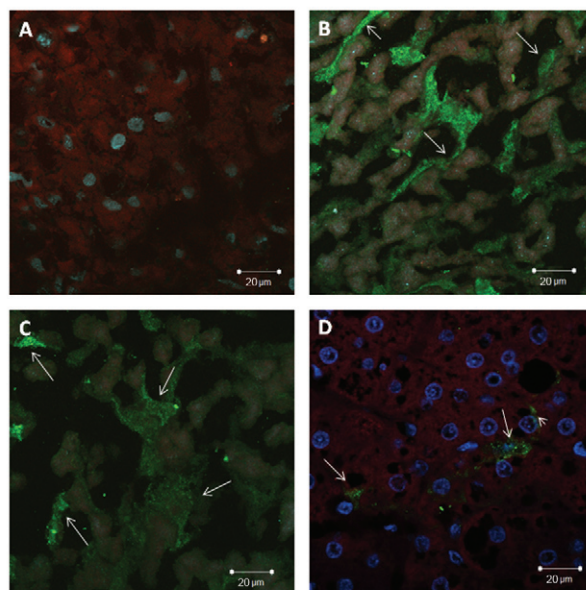
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gel electrophoresis; its detected molecular weight confirmed that it was in fact IgY. To further confirm this result, the binding specificity of our purified anti-HAV IgY to HAV antigens on VP1, VP2 and VP3 was characterised by WB (Towbin et al. 1979) and in vitro neutralisation assay. Antibody levels were also titrated.

To be able to use IgY as a diagnostic, an additional purification was required. The anti-HAV IgY that was purified from egg yolks by PEG was subjected to thiophilic adsorption using a HiTrap IgY Purification HP column according to the manufacturer's instructions (GE Healthcare Bio-Sciences AB, Sweden). The purified IgY was dialysed against phosphate buffered saline (PBS) and the samples were concentrated using PEG.

HAV antigens were detected by IIF in histological sections of frozen liver samples taken from cynomolgus monkeys (*Macaca fascicularis*). These samples were obtained from Amado et al. (2010), who had subjected cynomolgus monkeys to experimental infection with the Brazilian HAV strain (HAF-203; GenBank AF268396) in a previous study. First, the frozen liver samples were embedded in resin Tissue-Tek® (Miles Inc Diagnostic Division, USA) at low temperatures (dry ice). Next, the sections were cut into 4- μ m-thick slices using a cryostat and they were then applied to slides, fixed with acetone at 4°C and frozen at -70°C. The slides were blocked with a solution containing 2.5% bovine serum albumin and 8% foetal bovine serum (FBS) for 120 min in a moist chamber at 37°C. The slides were washed three times with PBS (pH 7.2). Subsequently, the tissue was incubated in a moist chamber at 37°C with the anti-HAV IgY (0.337 mg/mL) primary antibody at a dilution of 1:120 for 90 min. The slides were washed three times with PBS. Finally, the slides were incubated with goat anti-IgY IgG/Alexa Fluor® 488 (2 mg/mL) secondary antibody at a dilution of 1:1,200 (Molecular Probes®/Life Technologies™, USA) for 90 min in a dark chamber at 37°C. The slides were again washed three times with PBS. Evans blue dye was used to counterstain the histological sections and Slow Fade® glycerol with 4',6-diamino-2-phenylindole was added (Invitrogen™/Life Technologies™, USA). Images of the liver sections were obtained using a LSM Zeiss 510 Meta confocal microscope (Carl Zeiss, Germany). Additionally, the primary antibody was replaced with a nonspecific chicken immunoglobulin IgY (0.314 mg/mL) at a dilution of 1:120 to verify the specificity of staining in infected tissues. For comparative purposes, a commercial monoclonal anti-HAV mouse IgG (0.1 mg/mL) diluted at 1:20 (United States Biological, USA) was also tested, as described by Amado et al. (2010).

We next determined that our purified anti-HAV IgY bound specifically to viral antigens present in sinusoidal lining cells and to hepatocytes scattered throughout the liver parenchyma of infected monkeys (B, C in Figure). A control antibody was used to verify the specificity IgY and it did not bind to HAV in an infected liver biopsy sample; specific staining of antigen-antibody was not observed (A in Figure). Furthermore, no background or non-specific binding was visualised when the anti-HAV IgY



Hepatitis A antigen labelled with immunoglobulin Y (IgY) by indirect immunofluorescence with confocal microscopy of the livers from cynomolgus monkeys inoculated with hepatitis A virus (HAV). A: absence of fluorescence label cells using a nonspecific IgY in HAV infected liver (control section); B, C: anti-HAV IgY as a primary antibody binding specifically with viral antigens in cytoplasm of sinusoidal lining cells (SLCs) (arrows); D: commercial anti-HAV IgG as primary antibody binding specifically with viral antigens in hepatocytes (arrowhead) and cytoplasm of SLCs (arrows).

was used, indicating that its purification by thiophilic adsorption was efficient at removing potentially interfering molecules from solution. Indeed, our purified anti-HAV IgY presented more clear and evident results than commercial mouse IgG (D in Figure). The staining of viral antigens was weaker with IgG, even when the IgG concentration was two-fold higher than the IgY (0.005 mg/mL vs. 0.0025 mg/mL). Thus, IgY demonstrated superior effectiveness, as only half of the amount of IgY was needed to produce more satisfactory results than IgG.

In this study, for the first time, the ability to use IgY as an alternative and specific diagnostic of hepatitis A in liver sections has been demonstrated, reinforcing the importance of this approach. Such an approach was previously demonstrated for the detection of *Mycobacterium avium* subsp. in murine macrophages derived from bone marrow (Shin et al. 2009). Saniee et al. (2013) also proved the efficiency of using IgY to identify *Helicobacter pylori* via direct immunofluorescence. Additional studies have described using IIF to detect hypoxia-inducing factor-1 alpha in COS-7 monkey cells (Camenisch et al. 1999). Our results demonstrated the effectiveness of using IgY as a detection tool to facilitate the diagnosis of HAV by immunofluorescence. This work contributes to the dissemination of knowledge regarding IgY technology, which may enable it to play an even bigger role in research, diagnostics and immunotherapy.

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