

Studies on Diagnostic Applications of Egg Yolk Antibodies against *Bordetella bronchiseptica*

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Summary

The present paper describes the methods for producing egg yolk antibodies against *Bordetella bronchiseptica*. In comparative experiments, avian and mammalian antibodies were applied in diagnostic test systems. The results of these investigations show the possibility of substituting mammalian antibodies for IgY in enzyme immunoassay and indirect immunofluorescence.

Keywords: egg yolk antibody, IgY, *Bordetella bronchiseptica*, enzyme immunoassay, immunohistochemistry

Zusammenfassung: Untersuchungen zur diagnostischen Anwendung von Dotterantikörpern gegen *Bordetella bronchiseptica*.

Die vorliegende Arbeit beschreibt die Methoden zur Herstellung von Dotterantikörpern gegen *Bordetella bronchiseptica*. In diagnostischen Testsystemen wurden aviäre und mammäre Antikörper vergleichend eingesetzt. Die Ergebnisse dieser Untersuchungen zeigen die Möglichkeit des Ersatzes von Säugerantikörpern durch Dotterantikörper im Enzymimmunoassay und in der indirekten Immunfluoreszenztechnik.

1 Introduction

Polyclonal antibodies are typically isolated from the serum of immunised mammals such as rabbits, goats, or sheep. In contrast, the induction of antibodies in laying hens, with the egg yolk as antibody source, is a simple and practicable alternative to traditional polyclonal antibodies. The egg yolk antibodies are a specific and sensitive tool for detection, characterisation, quantitation, and localisation of different infectious agents. Specific avian antibodies are widely used for diagnostic tests and analytical systems, especially immunological standard methods.

Bordetella (B.) bronchiseptica is a small, gram-negative, motile, aerobic coccobacillus that causes respiratory diseases in pigs, dogs, cats, horses, and some laboratory animals. The diseases are economically important, because infected animals are predisposed to secondary bacterial or viral infections. Different vaccination programs were developed against *B. bronchiseptica* infection and/or against the complex of respiratory diseases, in particular against the atrophic rhinitis of swine.

The detection of the whole bacteria or specific antigens of *B. bronchiseptica* by using polyclonal antibodies is

one important part of established diagnostic tests.

The aim of the present study was the induction of antibodies against *B. bronchiseptica* in laying hens, the preparation of specific IgY from egg yolk, and the demonstration of applications of these egg yolk antibodies in enzyme immunoassay and immunohistochemistry.

2 Materials and Methods

2.1 *Bordetella bronchiseptica*

The *B. bronchiseptica* strain VST 19 I, an isolate from a pig with atrophic rhinitis, was used in our experiments (Krüger and Horsch, 1982 a,b). The strain was cultured on ACMM agar (Simmons citrate agar supplemented with glycerol, starch and nicotinic acid) under standard conditions (Kasprzak et al., 1994). Following 48 h of incubation at 37 °C the bacteria were harvested, washed in 0,15 mol/l NaCl solution, and centrifuged (400 xg, 20 min, 4 °C). The *B. bronchiseptica* strain was used in two forms for induction of egg yolk antibodies, as whole cell antigen and after urea extraction (Schilow and Nossack, 1988).

2.2 Immunisation procedure

The antigens were homogenised (1:1) with Freund's complete adjuvant for the initial antigen application or with Freund's incomplete adjuvant for the

booster injections. These antigen-adjuvant mixtures (0,5 ml) were injected into the pectoral muscle on both sides of laying hens (22 weeks old). The chickens were boosted after 40, 80 and 145 days in the same manner. The eggs were collected daily over the whole immunisation period and were stored at 4 °C until separation of the IgY from the yolk.

2.3 Antibody extraction

The egg yolks containing the specific antibodies were separated from the egg white and were diluted (1:5) with deionised water (pH 7,2). The yolk-water mixture was homogenised and frozen three times at -20 °C. Following this, the material was thawed and centrifuged (1500 xg, 20 min, 4 °C). Purification of IgY from the clear supernatant was done by a two-step precipitation procedure using saturated ammoniumsulfate solution and the so-called „BATCH“-extraction using Bakerbond Abx-matrix (J.T.Baker B.V., Germany) as described by Fischer et al. (1996). The IgY was shown to be greater than 90 % pure by SDS-PAGE analysis (Fischer et al., 1996). Purified IgY was frozen at -70 °C after protein concentration measuring (Peterson, 1979).

Rabbit immune sera against *B. bronchiseptica* antigens were a gift of the

Institute of Microbiology and Control of Animal Diseases (Veterinary Faculty, Berlin). The isolation of the IgG fraction from the rabbit sera was carried out as described by Ambrosius and Lippa (1987).

2.4 Detection of the specific egg yolk antibodies by an enzyme immunoassay

Microtiterplates (Nunc, immunoplate F 96, Polysorp) were coated with 50 µl per well of *B. bronchiseptica* antigen at a concentration of 10 µg/ml in carbonate/bicarbonate buffer (pH 9,6), over night at 4 °C. After washings, 50 µl of the avian antibody dilution were added in each well. The antibodies were diluted in so-called incubation buffer (8,0g NaCl; 0,2g KH₂PO₄; 2,9g Na₂HPO₄ × 12 H₂O, 0,2g KCl; 0,5ml Tween 20; Aqua dest. ad 1000,0 ml, pH 7,4, plus 10 % Gelafusal / Serumwerk Bernburg, Germany). Following an incubation of 1h at 37 °C, the plates were washed three times and 50 µl/well POD-labeled anti-chicken IgG (SIGMA, Immunochemicals) were added (1:20.000 in incubation buffer). The plates were incubated for 1 h at 37 °C. Following this, the plates were washed and 50 µl/well substrate chromogen solution (↓ ↓) was added. The reaction was stopped with 1M H₂SO₄ (50µl/well) after 20 min. The absorbance was measured at 492 nm using a SLT-reader system (SLT Labinstrumments, Germany).

The titre of the specific IgY was defined as the dilution of the antibody at which the absorbance/optical density

(OD) was threefold higher than the OD of an unspecific control antibody.

2.5 Enzyme immunoassay to detect *B. bronchiseptica* antigen

We established an enzyme immunoassay to detect *B. bronchiseptica* antigen. In this assay we compared the avian and the mammalian antibodies. The test principle and procedure are shown in fig. 2.

We used the avian antibodies as catch-antibody or as first detection antibody in comparison to the rabbit antibody.

This test variant was also used for the specificity comparison of the rabbit and avian antibodies.

Test variant A.:

The wells of an microtiterplate were coated with 1,0 µg/ml of purified avian antibody, and incubated over night at 4 °C. After the washing step, the plates were blocked with incubation buffer and incubated for 30 min at 37 °C. Following this, the *B. bronchiseptica* antigen was added in various dilutions for 2 h at 37 °C. Plates were washed and 50 µl per well of the specific rabbit antibody (1:1000 in incubation buffer) were pipetted in each well. Following this step, 50 µl/well of POD-labeled anti-rabbit IgG (SIGMA, Immunochemicals) was added. Finally, we added 50 µl substrate solution (5 mmol/l o-phenyldiamin, 5 mmol/l H₂O₂ in citrate buffer, pH 5,0) into the wells. The reaction was stopped with 1M H₂SO₄ after 20 min. Absorbance was read at 492 nm.

Test variant B.:

The microtiterplates were coated with 1,0 µg/ml of rabbit antibody and incubated over night at 4 °C. After washings and the blocking step, the *B. bronchiseptica* antigen dilutions were added (50 µl/well). Following this, 50 µl/well of the purified egg yolk antibody (1:1000 in incubations buffer) was added. After the incubation and the washing procedure, 50 µl of POD-labeled anti-chicken IgG (SIGMA, Immunochemicals) was pipetted in each well. Following this we added 50 µl of substrate solution into the wells. We stopped the reaction with 1M H₂SO₄ after 20 min. Absorbance was read at 492 nm using.

2.6 Indirect immunofluorescence (IF)

After acetone fixation (10 min) bacterial cells were incubated with different specific antibodies directed against *B. bronchiseptica* antigens. The rabbit anti-*B. bronchiseptica* antibody was diluted 1:20 in PBS and incubated for 30 min at room temperature. A FITC-labeled anti-rabbit IgG (Dianova), diluted 1:50 in PBS, was added onto the slides after three washing steps with PBS. The slides were incubated 30 min at room temperature.

Specific egg yolk antibodies against *B. bronchiseptica* and an avian antibody against tetanus toxoid (negative control) were diluted 1:20 in PBS. The

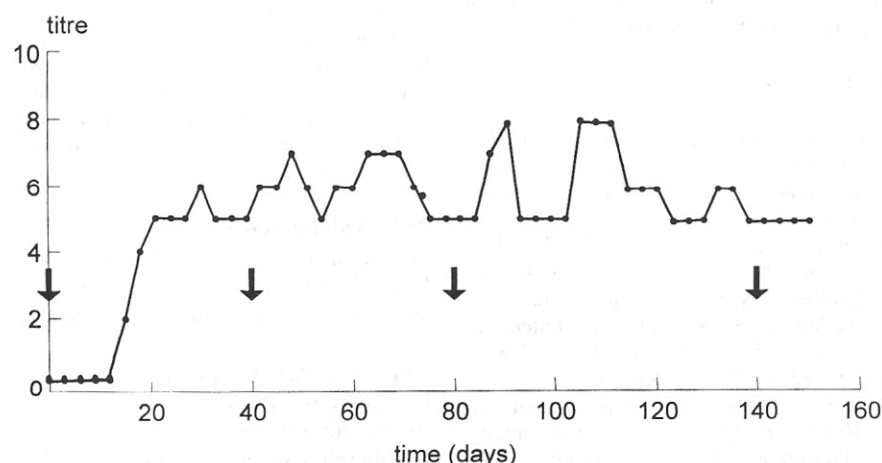


Figure 1: Time-curve of antibody titre development after immunisation with *B. bronchiseptica* antigen. ↓ = antigen application

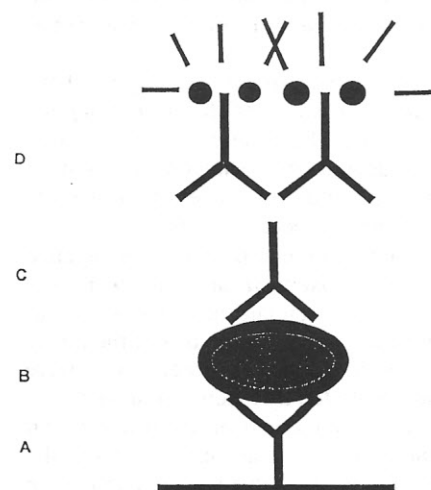


Figure 2: Enzyme immunoassay to detect *B. bronchiseptica* antigen
A = catch-antibody
B = antigen
C = detection antibody
D = anti-species- antibody (POD-labeled)

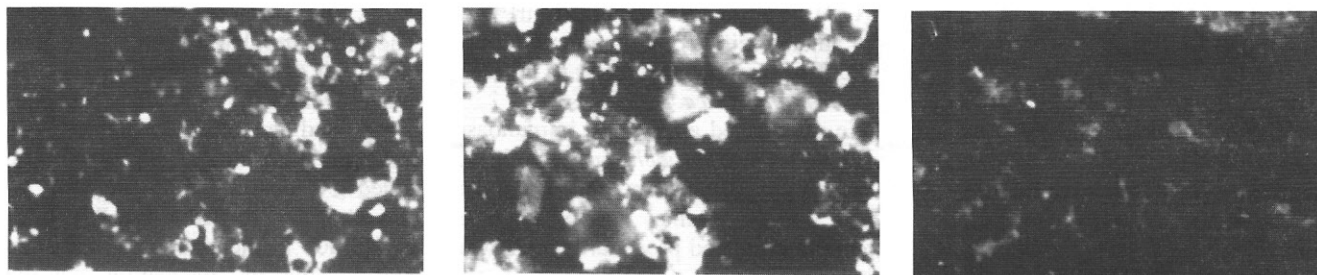


Figure 3: Suspended bacterial cells stained by the IF-technique ($\times 100$). a) reaction pattern after staining with egg yolk antibody ; b) reaction pattern after staining with rabbit antibody; c) after staining with an unspecific avian antibody (anti-tetanus toxoid)

avian antibodies or PBS (unspecific control) were added to the fixed antigen for 30 min at room temperature. After washings with PBS the slides were incubated with FITC-labeled anti-chicken IgG (SIGMA Immunochemicals; 1:32 diluted in PBS).

3 Results and Discussion

Egg yolk antibodies are a real alternative to mammalian antibodies in several diagnostic and analytical systems based on immunological reactions (Schade et al., 1991). Many studies have demonstrated the induction, preparation and application of specific avian antibodies against various infectious antigens (Hiepe et al., 1988; Graewskaja et al., 1988).

We described the production of egg yolk antibodies and the use of these immunological tools in enzyme immunoassay and in immunohistochemistry.

We have immunised laying hens with two kinds of *B. bronchiseptica* antigens. The time-curve of antibody titre development showed a continuous increase or a stable level of the specific IgY titre, respectively (fig. 1).

Large amounts of antibodies against *B. bronchiseptica* antigens were extracted by standard methods with simple equipment. The results of the purity analysis of our IgY preparations were demonstrated by Fischer et al. (1996).

The comparison of avian and rabbit antibodies in the tests showed the possibility of substituting mammalian antibodies by IgY in enzyme immunoassay and immunohistochemistry.

We could use the purified egg yolk antibodies in enzyme immunoassay as catch antibody or as first detection antibody to identify the *B. bronchiseptica*

antigens (fig. 2). Furthermore we could demonstrate a good correlation between the avian and mammalian antibodies in the enzyme immunoassay for detection of the urea extracted antigen (not shown). The egg yolk antibody shows the same high sensitivity and specificity as the rabbit antibody. But to identify the whole bacterial antigen in enzyme immunoassay the avian antibodies showed an insufficient sensitivity. The avian antibody is not suitable for the detection of the whole bacterial antigen in the present condition. In present experiments we examine different IgY preparations and various assay conditions to improve the diagnostic application of these avian antibodies.

In the immunohistochemical investigations we could demonstrate the absolute substituting of mammalian antibodies by IgY (fig. 3).

In present studies we investigate section material to detect *B. bronchiseptica* antigen.

In the future we want to produce egg yolk antibodies against subunits of bacteria, such as lipopolysaccharides.

References

- Ambrosius, H. and Lupp, H. (1987). *Immunhistochemie. Grundlagen und Techniken*. 229 pp. Berlin: Akademie Verlag.
- Fischer, M., Hlinak, A., Montag, Th., Claros, M., Schade, R. and Ebner, D. (1996). Vergleich von Standardmethoden zur Präparation von Dotterantikörpern. *Tierärztl. Praxis* 24, 411–418.
- Graewskaja, N. A., Kusow, J. J. and Donets, M. A. (1988). Eigelb immuner Hühner als Quelle für die Gewinnung von Immunglobulinen für die Diagnostik von Virusinfektionen. *Mh. Vet. Med.* 43, 677–680.
- Hiepe, F., Schwarz, G. and Hiepe, Th. (1988). Untersuchungen zur Antikörpergewinnung aus dem Eigelb immunisierter Hühner. *Mh. Vet. Med.* 43, 680–682.
- Kasprzak, H., Rehak, E. and Krüger, M. (1994). Influence of culture conditions on expression of antigens in *Bordetella bronchiseptica*. *J. Vet. Med. B*, 41, 645–653.
- Krüger, M. and Horsch, F. (1982). Untersuchungen zur Differenzierung von *Bordetella-bronchiseptica*-Stämmen. 1. Mitteilung: Nachweis des hitzelabilen Exotoxins und des Hämagglutinins. *Arch. exp. Vet. med.* 36, 691–698.
- Krüger, M. and Horsch, F. (1982). Untersuchungen zur Differenzierung von *Bordetella-bronchiseptica*-Stämmen. 2. Mitteilung: Nachweis der protektiven Eigenschaften ausgewählter Stämme im Kreuzschutzversuch. *Arch. exp. Vet. med.* 36, 699–702.
- Peterson, G. L. (1979). Review of the folin phenol protein quantitation method of Lowry, Rosenbrough, Farr and Randall. *Analyt. Biochem.* 100, 201–220.
- Schade, R., Pfister, C., Halatsch, R. and Henklein, P. (1991). Polyclonal IgY antibodies from chicken egg yolk – an alternative to the production of mammalian IgG type antibodies in rabbits. *ATLA* 19, 403–419.
- Schilow, W. F. and Nossack, A. (1988). Einsatz des Ultraschall behandelten und kompletten *Bordetella -bronchiseptica*-Antigen im indirekten Enzymimmunoassay. *Arch. exp. Vet. med.* 42, 324–327.

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