Campylobacter fetus Diagnosis: Direct Immunofluorescence Comparing Chicken IgY and Rabbit IgG Conjugates

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Summary

In Argentina Bovine Genital Campylobacteriosis is routinely diagnosed by direct immunofluorescence test. Generally, the hyperimmune sera used for this test are obtained from rabbits and less often from goats. In this work, a chicken egg yolk immunoglobulin (IgY) extract was conjugated and its ability to detect campylobacters with the regular conjugate prepared with rabbit sera was comparatively evaluated. Both conjugates were independently evaluated by two laboratories, named "Azul" (Lab A) and "Balcarce" (Lab B). Animals were immunised with formalin inactivated Campylobacter (C.) fetus cells. Chicken IgY and rabbit IgG were conjugated with fluorescein isothiocyanate and used to comparatively examine strains of C. fetus subspp., other Campylobacter spp. and different bacterial species. Both conjugates had a high percentage rate of detection for C. fetus. IgY had less background due to unspecific fluorescence than IgG. IgY is a cheap, bloodless and very productive method. IgY can replace mammal immunoglobulins for C. fetus diagnosis.

Keywords: immunofluorescence, egg yolk, IgY, IgG, Bovine Genital Campylobacteriosis Campylobacter fetus Zusammenfassung: Campylobacter fetus Diagnostik: Vergleich der Effektivität von fluoreszenzmarkierten Huhn- (IgY) und Kaninchen- (IgG) Antikörpern im direkten Immunfluoreszenztest

Die bovine genitale Campylobacteriosis wird in Argentinien routinemäßig durch einen direkten Immunfluoreszenztest diagnostiziert. Im Allgemeinen werden hierfür Hyperimmunsera vom Kaninchen, seltener von der Ziege verwendet. In der vorliegenden Arbeit wurde die Leistungsfähigkeit markierter IgY-Antikörper (präpariert aus Eidotter) vom Huhn mit den üblicherweise eingesetzten Antikörpern vom Kaninchen verglichen. Hierzu wurden sowohl Hühner als auch Kaninchen mit Formalin-inaktivierten Campylobacter (C.) fetus Zellen hyperimmunisiert. Die erhaltenen Antikörper wurden mit Fluoreszein-Isothiocynat markiert und zur Detektion von C. fetus subspp., anderen C. spp. sowie davon unterschiedlichen Bakterien eingesetzt. Eine Evaluierung beider Konjugate erfolgte unabhängig in zwei unterschiedlichen Laboratorien, bezeichnet als "Azul" (Lab. A) und "Balcarce" (Lab. B). Beide Konjugate detektierten zu einem hohen Prozentsatz C. fetus. Allerdings zeichneten sich die IgY-Konjugate im Vergleich zu den IgG-Konjugaten (Kaninchen) durch eine deutlich geringere unspezifische Hintergrundfärbung aus. Somit ist die IgY-Technologie auch für diesen Einsatzzweck eine tierschonende, kostengünstige und sehr produktive Methode. Auf der Basis der Ergebnisse dieser Studie kann konstatiert werden, daß für die Diagnose von C. fetus herkömmliche Kaninchen-Antikörper durch IgY-Antikörper ersetzt werden können.

1 Introduction

Bovine Genital Campylobacteriosis (BGC) is a disease generally caused by *Campylobacter* (*C.*) *fetus* subspecies (ssp.) *venerealis* and occasionally by the ssp. *fetus* (Dekeyser, 1984; MacLaren and Agumbah, 1988). This disease is characterised by infertility and sporadic abortion (Garcia et al., 1983) and because natural breeding is commonly used, it is a widespread disease among herds from Argentina (Villar and Spina, 1982; Russo et al., 1998; Boullon et al., 2000). Due to practical and economical reasons, traditionally the control of this disease mainly relies on the examination and vaccination of bulls, also the carrier cow can maintain the infection from one breeding season to another (Cipolla et al., 1994). At present its diagnosis is usually performed in males employing, as a screening test on preputial smegma, the direct immunofluorescence (DIF) test. Bacteriological culture of BGC is an alternative method, though its cost and complexity determines that only few laboratories with appropriate infrastructure can routinely perform it. DIF and culture can both be used to diagnose samples of preputial smegma, cervico-vaginal mucus and material from aborted foetuses.

In Argentina, mainly during 1983-92 the number of veterinary laboratories working on this disease has increased due to the implementation of a National Sanitary Plan to control BGC and trichomoniasis (Terzolo et al., 1992; Dillon et al., 1997). At present, about 30 laboratories are engaged in the control of BGC and they routinely employ the DIF test in bull's screening (Villar and Spina, 1982; Russo et al., 1998; Boullon et al., 2000). As a direct consequence of the implementation of this plan, the percentage of BGC infection in meat herds decreased from around 50% in 1983 (Cipolla et al., 1997) to around 15-18% in the period 1997-1999. This BGC infection decrease can be essentially attributed to the successful diffusion and implementation of DIF screening test to detect infected bulls during 16 years. From 1989 onwards, the Scientific Commission on Bovine Venereal Diseases, dependent on the Argentine Association of Veterinarians of Diagnosis Laboratories, recommended to the professionals in charge of the laboratories to participate each year in a voluntary official control of their DIF technique and conjugates. The professional staff of the National Institute of Agricultural Technology (INTA), Experimental Station of Balcarce, annually conducted the quality control. The participating laboratories have annually carried out the control tests from 1983 until today. During all these years, the number of participant laboratories varied from 6 to 19 and they have used from 7 to 3 different conjugates. All conjugates were elaborated using mammal hyperimmune sera, mainly prepared from rabbits and only once a conjugate was elaborated from a goat serum. The diagnosis efficiency detected in these controls, using formalin fixed bacterial antigens and preputial washings, improved through the years from 78% in 1991 up to 98% in 1996 and the following years.

Considering that in our country conjugates for DIF tests are essential for the control of BGC and a significant number of analyses have to be annually carried out by different laboratories, quite a lot of rabbits have to be killed every year to prepare the conjugates. As the specific hen antibodies concentrate in the egg yolk at a title similar to blood serum (Schade et al., 1996 and 2000) and separation egg yolk immunoglobulins (IgY) from the lipids (Schwarzkopf and Thiele, 1996; Schade et al., 2000) is now available, replacement of rabbits by hens is an alternative method to be implemented. In fact, the hen is able

to produce eggs containing specific IgY against a great variety of bacterial antigens: C. jejuni and C. coli (Chandan et al., 1994); Escherichia coli (Yokoyama et al., 1992; Ikemori et al., 1993; Tanimoto et al., 1995); Salmonella enterica serovar Enteritidis (Peralta et al., 1994; Terzolo et al., 1998); Brucella abortus (Lösch et al., 1986) and Edwarsiella tarda (Gutiérrez et al., 1993). Besides, chicken IgY has been used to prepare fluorescent conjugates for the diagnosis of rabies, some avian viral diseases (Staak, 1996) and Bordetella bronchiseptica (Hlinak et al., 1996). Thus, a work to evaluate the ability of IgY conjugates to detect C. fetus sspp. strains was undertaken.

This study was carried out to induce antibodies against *C. fetus* sspp. strains in laying hens and rabbits, to extract specific IgY from egg yolks and to prepare and evaluate these two immunofluorescent conjugates by two independent laboratories.

Unfortunately, for this study it was not possible to use hen keeping equipment according to the standard usual in some European countries (at least in public institutions). Therefore, the improvement of keeping conditions is one aspect of a joint IgY-project between Argentina and Germany which started in this year.

2 Animals, materials and methods

The rabbits and chickens were both separately kept using the available standard field rearing systems. All animals were reared in cages to prevent coccidiosis and other diseases. The rabbits were individually allocated in outdoor cages. Rabbit cages are 70 cm long, 80 cm wide and 50 cm high; they have cement roof and walls, wired floor and a double plastic mesh 2 meters over the cages to protect the animals from the sunlight. The hens were caged indoors using the available commercial wired batteries (41 cm x 41 cm), originally designed to keep three hens per cage. All hens were individually caged so as to have plenty of room. The construction of the cages allowed the hens to have optical and acoustical contact. Each cage has devices to feed and provide drinking water to the animals. All these cages are elevated from the floor so as the faeces drop on a cement floor. These faeces were daily collected, and the floor cleaned and disinfected to minimise the risk of infection with avian pathogens.



Animals were fed balanced food specifically formulated for rabbits and laying hens. Balanced foods were free from antibiotics, coccidiostats and had only vegetal ingredients, avoiding either meat or fishmeal. Batches of this balanced food were tested for detection of aflatoxins B1, B2, G1 and G2, ochratoxin A, T-2 toxin and zearalenone and found to be negative. Bacteriological analyses were made to check absence of coliform bacteria in water and Salmonella in water and food. All animals received water ad libitum. Rabbits were fed ad libitum the balanced food supplemented with alfalfa (Medicago sativa) and hay and also have straw provision for bedding. Each hen received daily 100 grams of balanced food supplemented with ground seashell. A lighting programme to increase egg production was adapted and the number of eggs was recorded daily.

To obtain specific serum antibodies, two 8-week-old New Zealand rabbits received three 0.5 ml subcutaneous applications of a vaccine at days 0,33 and 72. Fifteen days after the last injection both rabbits were bled.

To obtain specific egg yolk antibodies, eight Golden Comet layers were immunised with two vaccines. Five layers were immunised with vaccine 1 and the other three hens with vaccine 2 (vide infra). All hens were given two 0.5 ml doses into the pectoral muscles, separated by a 15-day interval. Nineteen days after the second dose, all eggs were collected daily during 3 days and stored at 4°C.

2.1 Bacterial strains

A total of 67 bacterial strains belonging to different genera and species were evaluated: Campylobacter (57 strains) including C. fetus subspp. (53) and other Campylobacter (4); Bacillus sp. (1); Brucella abortus (1); Proteus sp. (1); Actinomyces pyogenes (1): Escherichia coli (1): Salmonella enterica serovar Dublin (1); Staphylococcus aureus (1); Pseudomonas aeruginosa (1); Streptococcus sp. (1) and Klebsiella pneumoniae (1). Out of the 53 strains of C. fetus subspp., 35 were C. fetus fetus (34 regional isolates and one international reference strain), 11 C. fetus venerealis (10 regional isolates and one of international reference strain) and 7 C. fetus venerealis biotype intermedius (all regional isolates).



Each one of these strains was preserved in liquid nitrogen at -196° C. After thawing, sub-cultures on Columbia agar, supplemented with 7% of bovine blood were made. Incubation according to the temperature and atmosphere requirements of the different genera was carried out. Once an abundant growth was obtained, the strains were suspended in 1% formalin sterile PBS, pH 7.2. The concentration of each suspension was standardised to McFarland's scale number 0.5 (1.5 x 10⁸ cells per ml).

2.2 Vaccines for rabbits

The vaccine was an equivalent mixture of five *C. fetus* strains: *C. fetus* venerealis NCTC 10354, *C. fetus* venerealis INTA 371, *C. fetus* venerealis INTA 395, *C. fetus* fetus NCTC 10348, *C. fetus* fetus INTA 96/567 and *C. fetus* fetus NCTC 5850. The final concentration of the antigen mixture was adjusted to 6 x 10⁹ cells/ml by means of the McFarland scale. One part of antigen, in a vehicle of 0.5% (v/v) formalin PBS pH 7.2, was mixed and emulsified with one part of Freund's complete adjuvant.

2.3 Vaccines for chickens

Vaccine 1 was prepared with an equivalent proportion of three regional strains of C. fetus venerealis, C. fetus venerealis biotype intermedius and C. fetus fetus. Vaccine 2 contained equal parts of 5 strains, including the three previously mentioned regional strains and two other regional strains of the subspecies venerealis and fetus. Both vaccines were inactivated with 0.5% formalin and then 20% (v/v) aluminium hydroxide gel containing 2% (w/ v) of aluminium oxide was added as adjuvant. The final live bacterial concentration, determined by the method of Miles et al. (1938), varied from 1 to 6 x 109 CFU (average concentration per strain was around 1.3 x 10⁹).

2.4 Rabbit serum IgG extraction and conjugation

The proteins were separated from the serum by precipitation with 70% (w/v) of $(NH_4)_2SO_4$. This solution was added at a volume identical to the original serum. After precipitation, the mixture was centrifuged and the supernatant was discarded. The sediment was suspended in distilled water at the same volume of the

original serum. After the first (NH₄)₂SO₄ precipitation, this procedure was repeated twice. Finally, the sediment was dissolved in distilled water, using a volume of water equal to half of the original volume of the serum. The resulting globulins were dialysed against a 0.85% NaCl solution until no BaSO₄ precipitation was observed in the solution when a 40% (w/v) of BaCl, was added. The amount of proteins in mg was determined at 540 nm in a semiautomatic SEAC spectrophotometer model CH16 (Gornall et al., 1949). Globulins were conjugated with a solution of fluorescein isothiocyanate in Na, HPO, pH 9, at about 25-30 µg per mg of protein. Next the conjugate was centrifuged at 2,800 g during 20 minutes. Thereafter the supernatant was dialysed at 4°C, against PBS pH 7.6, until the dialysis solution remained clear. Finally 0.02% (w/v) N₂Na was added.

2.5 Chicken egg IgY extraction and conjugation

A dozen egg yolks from hens of both groups, free from their membranes and of albumin, were pooled together, mixed and homogenised (Schade, 2000). To obtain high purity IgY from fresh yolks, the polyethyleneglycol extraction method of Polson et al. (1980) was used (PROMEGA® kit "EGGstract IgY purification System"). Three precipitations were made according to the manufacturer's instructions. Afterwards the same procedure previously described for the rabbit serum was carried out, including two precipitations with 70% (NH₄)₂SO₄ (Schwarzkopf and Thiele, 1996), dialysis against PBS pH 7.6 and conjugation with fluorescein isothiocyanate. This extract was also preserved with 0.02% (w/v) N₂Na.

2.6 Participant laboratories

Two laboratories of veterinary diagnosis, located in the Southeast Region of Bue-

nos Aires Province, carried out this work. These laboratories were the private Laboratory Azul (Lab A) and the official Laboratory INTA Balcarce (Lab B). Both laboratories routinely use the DIF technique to detect *C. fetus* in bulls.

2.7 Immunofluorescence technique

The technique used by both laboratories was previously described (Dufty, 1967) except for the fixation step at Lab A. In this laboratory the procedure was modified fixing the slides by passing them 3 times slowly through the flame (Kita et al., 1966). Then, slides were incubated overnight at 37°C. Conjugates were diluted at the 1:20 with PBS, pH 7.2.

2.8 Evaluation of the conjugates

Both laboratories used the same method to evaluate the conjugates. Each laboratory worked with aliquots of the same formalinised antigens and twice two different operators observed each strain. A result was considered to be positive only if fluorescence was detected together with morphology compatible to the C. fetus. Different degrees of fluorescence were observed and recorded as follows: weak: +; good: ++; very good: +++. Strains were considered to be negative either if they did not show any fluorescence or the morphology did not correspond to C. fetus. Lab A and Lab B respectively used Carl-Zeiss and Nikon epifluorescence microscopes. Both microscopes worked under mercury vapour lamp.

3 Results

C. fetus strains were detected at a high rate by both conjugates, although more strains were detected by means of the rabbit conjugate (see Table 1). Using the IgY conjugate in Lab A only one *C. fetus* strain could not be detected whereas in Lab B two strains were missed. On the other hand, all *C. fetus* strains examined were

Tab. 1: C. fetus Lab A and B detection rates comparing chicken and rabbit conjugates

Lab	Chicken IgY N° and % of strains detected/evaluated	Rabbit IgG N° and % of strains detected/evaluated		
А	52/53 (98,1)	53/53 (100)		
В	51/53 (96,2)	53/53 (100)		



detected when the rabbit conjugate was used.

When these data were analysed comparing the different *C. fetus* subspecies (see Table 2), both laboratories failed to detect a single identical *C. fetus fetus* regional strain and only Lab B skipped the detection of a reference strain of *C. fetus venerealis* NCTC 10354. could greatly reduce the number of *Campylobacter* cells. Contamination of preputial samples is more frequent than those coming from vaginal mucus or aborted foetuses (Campero et al., 1993). DIF have the advantage to allow a rapid diagnosis, even in the presence of heavy contaminant flora of the preputial environment. Therefore the DIF technique is

Tab. 2: *C. fetus* subspp. Lab A and B detection rates comparing chicken and rabbit conjugates. Cfv: *C. fetus veneralis*, Cfvi: *C. fetus veneralis* biotype *intermedius*, Cff: *C. fetus fetus*

Lab	Chicken IgY Nº and % of strains detected/evaluated Cfv* Cfvi** Cff***			F № an deteo Cfv	Rabbit IgG Nº and % of strains detected/evaluated Cfv Cfvi Cff		
A	11/11 (100)	7/7 (100)	34/35 (97,1)	11/11 (100)	7/7 (100)	35/35 (100)	
В	10/11 (90,9)	7/7 (100)	34/35 (97,1)	11/11 (100)	7/7 (100)	35/35 (100)	

Using both conjugates, none of the other 14 bacterial species examined, all different from *C. fetus*, showed any fluorescence. Therefore the specificity of the chicken and rabbit conjugates was 100% at both laboratories.

Interestingly, in comparison with the IgG conjugate, all antigens analysed with the IgY conjugate showed lower unspecific background fluorescence.

4 Discussion

Bacteriological culture and DIF have been considered traditional methods for *C. fe*tus diagnosis. Using these techniques, the detection rates differ according to the reports of different authors. Thus, for culture detection, percentages near to or higher than 90% have been registered (Ardrey et al., 1972; Palladino and Campero, 1983; Hum et al., 1994) whereas for DIF the values have oscillated between 60% (Philpott, 1968) and 90% or even more (Mellick et al., 1965; Dufty, 1967).

When the isolation technique is used, it is essential to control the overgrowth of *C. fetus* by contaminant bacteria, such as *Pseudomonas* and *Proteus* genera. To avoid contamination, filtration of samples may be done, although this procedure a good procedure for the diagnosis of BGC in bulls, although it critically depends on the employment of reliable conjugates. These conjugates have to be specific and sensitive enough to respectively avoid detection of false positive and negative results. To that end, it is required to have both excellent immunofluorescent conjugates and high-quality optic microscope equipment, so as to achieve maximum sensitivity and specificity. In Argentina, conjugates for the BGC diagnosis have been commercialised for many years, being basically elaborated in mammals, and mainly in rabbits, following pre-established standard protocols (Mellick et al., 1965; Philpott, 1968).

This work compares the performance of a commercial rabbit conjugate, routinely used for diagnosis in Argentina, with an IgY conjugate particularly prepared for this experimental study. It was demonstrated that usage of incomplete Freund's mineral oil adjuvant in hens generally provides a better level of specific antibodies due to production of sterile abscesses, which increases the depot effect (Schade et al., 2000). In this work we used aluminium hydroxide gel adjuvant because it was ready available in our laboratory and, in our experience, it produces a good immune response with bacterial antigens. Future work has to consider also the usage of Freund's adjuvant by subcutaneous route (Schade et al., 2000).

The sensitivity of both conjugates was high, being 100% for the rabbit conjugate and 96.2% - 98.1% for the IgY conjugate. These differences may be due to: usage of different optic equipment in both laboratories, loss of antigens during subcultures, antigenic differences among the strains, and chiefly the inclusion of different strains in the vaccines for rabbits and in hens. Future work has to include several representative regional isolates in the elaboration of vaccines. In this work, when a C. fetus fetus regional strain was not included in the vaccine for hens, the IgY conjugate consistently skipped detection of this strain. This DIF negative result demonstrates antigenic difference among C. fetus strains. This phenomenon, which appears infrequently, was also recorded with the rabbit commercial antisera used in diagnosis cases in Argentina. It was demonstrated that the surface protein antigens of C. fetus, conforming the microcapsule, could be lost by several subcultures (Winter et al., 1978; Cipolla et al., 1992), so next works have to consider not only the selection of adequate strains but also their proper maintenance.

Other factors, such as dexterity of the operator in the reading of the slides and dilution of the conjugate are omitted, since our trained technicians have been performing this routine diagnosis task during over 20 years and all conjugates were equally diluted.

These preliminary results support future work, taking into account the following items: use of identical bacterial antigens in the formulation of the vaccines for rabbits and hens, evaluation of a large number of strains and also to include samples from infected cattle.

In comparison with the IgG conjugate, a lower unspecific fluorescence was observed with the IgY conjugate in all the samples examined. This fact could be explained because the IgY conjugate was elaborated in hens, an animal which is phylogenetically more distant from the bovine species than the rabbit (Shade et al., 2000). This property of IgY conjugates is crucial when samples containing mammal organic material, such as smegma or preputial washings, are micro-

scopically observed. Therefore replacement of rabbits by hens could improve the quality of conjugates for the diagnosis of BGC and also may improve the DIF diagnosis in other bacterial diseases.

Thus, the use of hens to produce specific antibodies is a real alternative to the use of rabbits from a view of animal welfare, science and also economy.

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Linz 2000, Poster Nachtrag

Poster

Im Heft 1/2001 fehlte bei der Berichterstattung über Linz 2000 leider dieses Poster. *ALTEX* bittet um Entschuldigung.

Evaluation of Nutrition Media Derived from Human Blood Transfusion Units DFP, EC and BC for the *in vitro* Feeding of *pediculus humanus corporis* (Anoplura: Pediculidae)

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The laboratory rearing of the human body louse Pediculus (P.) humanus corporis, main vector of Rickettsia prowazecki, Borrelia recurrentis and Bartonella quintana, is unalterably required for efficacy tests of pediculicids. Beside the practised for decades feeding procedure of the body lice on arms of volunteers, Culpepper succeeded for the first time in 1948 in breeding these obligate haematophagous and stenoxene human parasites exclusively by blood feeding on rabbits. Until now, rabbits are used as substitute hosts for the laboratory rearing of P. humanus corporis. However, the performance of feeding lice on rabbits repeatedly gave occasion for criticism from point of animal welfare. Hence, there is a demand

for the development and evaluation of alternative methods for continuous laboratory rearing and mass breeding of the human body louse.

Experimental investigations on the *in vitro* feeding of *P. humanus corporis* of a rabbit-adapted laboratory strain showed that this Anoplura species are able regularly to feed on heparinised and stored at 4°C fresh blood of volunteers, as well as on whole blood resuspended from human blood transfusion units (superimposed CPD-SAGM-stabilisised erythrocyte concentrate unit - EC, superimposed deep frozen Aphaeresis-fresh plasma unit – DFP and a buffy coat unit – BC) that were offered through a Parafilm M[®] membrane (American National Can, Chicago). Thus,

the body lice were kept in laboratory over 9 generations by continued in vitro feeding with blood stored until use at -27°C. However, a developmental deceleration of 1 to 2 days from the first feeding of first stage larvae until their moult into imagines of in vitro fed lice compared to rabbit fed lice was observed. Screenings of 13 nutrition media (storage 4°C vs. -27°C) derived from EC, DFP and BC and of 5 nutrition media extracted from BC in continued up to 30 days feeding experiments indicated that superimposed media from BC: GFP in ratios of 2:3, 1:1 und 3:2 are proper for long time feedings. After the use of BC-derived nutrition media P. humanus corporis showed the same developmental time from first blood uptake of first stage larvae to their moult into imagines as lice fed on the rabbits. This study demonstrates that P. humanus corporis can be reared by membrane feeding technique. The in vitro feeding of human lice offers the possibility of breeding these pediculids in considerateness to animal welfare for studies on lice control and efficacy tests of insecticides as well as for molecular biological and physiological studies. On the other hand this in vitro method enables to offer the lice defined and standardised nutrition media for advanced studies.