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ABSTRACT

The use of immunoperoxidase enabled us to determine the prevalence of infection by agents difficult to isolate and culture. We have detected rabbit respiratory pathogens in 45 of the 51 lungs belonged to animals with clinical respiratory disease (88%) and in 13 of 33 animals with no apparent pulmonary disease (39%). In rabbits with clinical respiratory disease, *Mycoplasma spp* was found in 43.1% of the lungs, followed by viral infections: *Myxomatosis* (29.4%) and *Rabbit Haemorragic Disease* (RHD, 23.5%). These 3 pathogens were significantly more prevalent among rabbits with respiratory disease than healthy ones. Detection of *Chlamydia psittaci* and *Toxoplasma gondii* was relatively high, both in sick animals and in healthy ones, ranging between 7.8% and 18.1%. In uterus of sick animals the presence of *C. psitacci* (35,0%) stands out, followed by Mycoplasma spp (29,6%), MXV (29,6%), T. gondii (20,4%), RHD (4,3%) and L. interrogans (0,0%) has not any relevance.

INTRODUCTION

Myxomatosis and Rabbit Haemorragic Disease (RHD) are generally not considered to be frequent respiratory syndrome agents, although we know of the tropism of these viruses to lungs and reproductive tract (Carrasco et al. 1991, Chasey 1997). Something similar occurs with *C. psittaci*, *T. gondii* and *L. interrogans* (McOrist et al., 1987, Gallazzi 1993, Boucher & Nouaille, 1996).

In some farms, we have observed similar lesions to those caused by *Mycoplasma spp.* in other animals species lungs, and we applied the standard methods for cultivation of *Mycoplasma spp* in lungs obtaining several isolates of *Mycoplasma spp.* which, were labelled with *M. pulmonis* antibodies, and which were kept in the laboratory's strains bank (data not published). We are not currently aware of any reference about isolation or prevalence of Mycoplasma spp infection in farm or laboratory rabbits, although is known the importance of Mycoplasma pulmonis in respiratory processes in other rodents, such as laboratory and wild rats and mice (Cartner et al. 1995).

The aim of this work is to study the prevalence of infection by bacterial (*Leptospira interrogans*) viral (Rabbit Haemorragic Disease Virus, RHDV, and Myxomatosis virus, MXV), and parasitic agents (*Toxoplasma gondii*), as well as Chlamydia (*Chlamydia psittaci*) and Mycoplasmas in lungs and uterus of farm rabbits, affected or not by a clinical respiratory disease, or reproductive problems using immunocytochemistry as diagnostic tools.

MATERIAL AND METHODS

Animals and samples

To study respiratory problems 84 rabbits were received (1998-1999), 51 were sent with a clinical history of respiratory disease and 33 with a history of digestive or cutaneous diseases, but not respiratory "healthy rabbits". For reproductive problems, samples were received along 1999 and the pathogens studied were requested by our customers. The samples included animals, isolated organs (uterus) or cervical swabs (Amies medium) with detached cells.

Preparation of the samples

A sample of aproximately 2 cm^2 was taken from each lung or uterus and washed with PBS. Cells were obtained perfusing the lungs with PBS 0.01 M pH 7.2 using a hypodermic syringe, or cutting the uterus in a trypsin solution (Sigma, T-4549) 0,1 % in PBS 0,01 M pH 7.2. The obtainings cells were placed into 1.5 ml Eppendorf tubes, centrifuged at 3000 x g for 5 minutes and washed 3 times in PBS. In the swabs, desquamated cells were detached and individualized with gentle agitation in a trypsin solution (Sigma, T-4549) 0,1 % in PBS 0,01 M, pH 7.2.

Cell population was adjusted in all cases to 5 - 6 x 10^5 cells/ml in a Neubauer chamber and 10 µl of cell suspensions were placed on multi-sphere slides. Cells were fixed 30 minutes in a solution of 20% acetone in PBS 0,01 M, pH 7.2 containing 0.1% of Tween 80 and 0.05% Cas-block (Zymed, 008020).

The non-specific inhibitors and the endogenous peroxidase were minimized with a 10 postfixative minutes in 0.3% Hydrogen peroxide. After several washes in PBS-Tween, we applied 10 µl of the primary antibodies for 1 hour in a humidity chamber at 37ʃC. As a negative control of the test, we used antibody diluent (PBS 0,01 M pH 7.2).

Secondary antibodies conjugated with peroxidase were applied to the test samples and controls with the same incubation parameters as the first.

The resulting immune complexes were revealed with 9 amino-3 ethyl carbazole (AEC, Sigma A-6926) in Dimethylformamide (DMF, Sigma D-4254) and buffer acetate 50 mM pH 5, applied for 10 minutes at room temperature in darkness. The reaction was stopped and counterstained with Mayer Haematoxylin (Zymed 00-8001). The slides, mounted with GVA (Zymed 00-8000), were studied on light field microscope at 1000 amounts. The positivity criteria were established against a negative control, included for each sample on the same slide.

Antibodies

The used antibodies were: Specific anti *Chlamydia* Mab (Argene, Biosoft) anti *Toxoplama gondii* IgG Mab (Argene, Biosoft); anti *Mycoplasma pulmonis* PG34 polyclonal (PHLS Aarthus Collection, London); specific 1H7 Mab contrasted with the VP60 protein of the *Rabbit Haemorragic Disease Virus* (Ingenasa) and specific anti papilloma virus BPV-1 polyclonal (Dako, B-0580), anti *Leptospira interrogans* polyclonal (Sanofi).

As second antibodies, anti IgG rabbit Mab were used conjugated with peroxidase (Sigma A-9452, Mab clone GT-34) for *M. pulmonis and L. interrogans*, and recombinative Protein G HRPO (Sigma P-8170) for the rest of antibodies (RHD, MX,*T. gondii, C. psittaci*)

RESULTS AND DISCUSSION

The use of immunoperoxidase enabled us to detecting antigens in 45 of the 51 lungs of ill animals (88.2%) and in 13 of the 33 health animals (39.3%). Among the rabbits with clinical respiratory disease, the presence of *Mycoplasma spp.* in 43.1% of the affected lungs stands out (Boucher et al. 1999), followed by the viral infections: MXV (29.4%) and RHDV (23.5%). In these 3 diseases, we clearly observed a greater prevalence of infection among the group of ill animals with clinical conditions than among the "healthy" ones. Detection of *C. psittaci* and *T. gondii* in lungs was relatively high, both in sick and in healthy animals, and ranging between 7.8% and 18.1%. No differences in the detection of *C. psittaci* were found between groups, however, stand up the high presence of *T. gondii* in lungs classified as "healthy" (Table 1).

Table 1: Prevalence of pathogens detected by indirect immunoperoxidase in rabbits lungs with clinical respiratory disease and anatomopathological lesions, and in animals with no apparent lung disease.

Antigen	Unhealthy n = 51		Healthy n = 33	
	Positive	%	Positive	%
RHDV	12	23.5 %	1	3.0 %
MXV	15	29.4 %	1	3.0 %
C. *psittaci*	7	13.7 %	5	15.1 %
T. gondii	4	7.8 %	6	18.1 %
Mycoplasmas spp	22	43.1 %	1	3.0 %
No antigen detected	6	11.7 %	20	60.6 %

Associations between pathogens were frequent, even though no dominant association was observed, no animal was found with two viral agents (MXV - RHDV) or with both *C. psittaci* and *T. gondii*.

Among the samples from reproductive problems the presence of *C. psitacci* (35,0%) stands out, followed by *Mycoplasma spp* (29,6%), MXV (29,6*%), T. gondii* (20,4%). RHD (4,3%) and *L. interrogans* (0,0%) has not any relevance.

Table 2: Pathogens detected by indirect immunoperoxidase in rabbits uterus and cervical swabs**.**

Antigen	Positive/studied	%
RHDV	1/23	4,3 %
MXV	8/27	29,6%
C. psittaci	28/80	35,0%
T. gondii	11/54	20,4%
Mycoplasmas	8/27	29,6%
L.interrogans	0/33	0,0%

Immunoperoxidase enabled the direct identification of RHDV, MXV, *C. psittaci*, *T. gondii* and *Mycoplasma spp*. quickly and highly specific. Immunoperoxidase has been used for the experimental detection of *Bordetella bronchiseptica* in lungs of rabbits with interstitial pneumonia, (Uzal et al. 1990), in the confirmatory diagnostic of RHD (Carrasco et al. 1991), in *Mycoplasmas* diagnosis (Scanziani, 1998) and in models of *Chlamydia* infection (Fong et al. 1997), but we do not know if it is

being used as a routine diagnostic method in rabbits.

The high prevalence of *Mycoplasma spp*. in unhealthy animals with respiratory problems (43.1%) and very low prevalence in healthy ones (3%) stands out, which could be indicative of an important involvement in the respiratory process. The fact that, to date, no *Mycoplasma spp*. infections in rabbits have been diagnosed, in our knowledge, might be attributed to the difficulties to cultivate this microorganism. The first significant work (Boucher et al.1999) was carried on with our isolates. *Mycoplasma spp* needs special nutritional requirements and is not usually included in routine diagnostics of rabbit respiratory pathology. Cultivation is complicated if, as in the case of rabbit lungs, there is a high bacterial contamination charge (*P. multocida*, *B. bronchiseptica*, *S. aureus*). The presence of such bacteria might lead us to justify the lesions found, so the search for other possible agents such as Mycoplasmas which may act as initiators of lesions upon which bacteria subsequently settle is not considered.

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