Demonstration of Rat CAR Bacillus Using a Labelled Streptavidin Biotin (LSAB) Method

Jorge ORÓS*, Satoru MATSUSHITA†, José L. RODRÍGUEZ, Francisco RODRÍGUEZ, and Antonio FERNÁNDEZ

Department of Histology and Pathology, Veterinary Faculty of Las Palmas, 35016 Las Palmas de Gran Canaria, Canary Islands, Spain, and *Section of Laboratory Animals, National Institute of Radiological Sciences, 4-9-1 Anagawa, Inage-Ku, Chiba 263, Japan

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ABSTRACT. Immunohistochemical detection of rat CAR bacillus antigen in paraffin-embedded experimentally infected rat lungs, using an immunoperoxidase technique based on the labelled streptavidin-biotin (LSAB) method and 3-amino-9-ethylcarbazole (AEC) as substrate is described in this paper. The pattern of immunostaining was confined to the ciliated bronchial epithelium and the specificity of this technique was confirmed. The use of AEC as substrate was evaluated more efficient than diaminobenzidine (DAB). The usefulness of this immunoperoxidase technique for the detection of CAR bacillus in rats and its advantages compared to the indirect immunofluorescence (IF) are discussed. — KEY WORDS: CAR bacillus, immunohistochemistry, rat.


Cilia-associated respiratory (CAR) bacillus is a descriptive term for unclassified, gram-negative, motile, filamentous bacteria that colonize the respiratory epithelium of laboratory and wild rats [1, 4, 7, 9, 10, 18], mice [13], rabbits [8, 17], cattle [6], pigs [15], goats and lambs [3]. The bacteria are approximately the same length and diameter as cilia and lie amongst and parallel to cilia [3, 4, 8, 10, 15, 17, 18].

Recently analysis of the 16S rDNA sequence has indicated that CAR bacillus found in rats and rabbits are two different organisms, being CAR bacillus from rabbits most closely related to members of the genus Helicobacter [2], whereas CAR bacillus from rats is most related to the genus Flavobacterium [16].

In rats, CAR bacillus colonizes the ciliated epithelial cells of the respiratory tract and causes a marked peribronchial infiltration and hyperplasia of mononuclear cells that progresses with time, leading to chronic bronchitis [1, 4, 9, 12, 17, 18].

The infection in rats has been diagnosed by histological examination using silver staining techniques such as Warthin Starry (WS) [1, 4, 9, 18] or electron microscope examination of the affected respiratory tracts [4, 10–12]. Serodiagnosis of CAR bacillus infection by the indirect immunofluorescence assay technique (IFA) has been also reported [14]. Immunostaining methods as indirect immunofluorescence (IF) and avidin-biotin complex (ABC) methods to detect CAR bacillus antigen in rabbits, guinea pigs, Syrian hamsters and mice have been reported [17].

In this paper we describe the detection of CAR bacillus antigen in paraffin-embedded lungs of rats experimentally infected with different concentrations of CAR bacillus. An immunoperoxidase technique based on the labelled streptavidin-biotin (LSAB) method and 3-amino-9-ethylcarbazole (AEC) as substrate was carried out.

Specific hyperimmune sera raised in specified-pathogen-free (SPF) male BALB/c mice against the SMR strain of CAR bacillus isolated from a naturally infected Wistar/Ms rat [10] were used. Antibody titers were estimated as more than 1:640 by the IFA technique. Polyclonal antibodies raised in rabbits against Mycoplasma pneumoniae, Salmonella sp. and Pasteurella sp. were used as negative controls.

Lung tissues from SPF male Wistar/Ms rats intranasally inoculated with 0.1 ml of an inoculum containing 8.5 x 10⁷/ml of the CAR bacillus SMR strain [12] and rats experimentally infected with 0.1 ml (7.5 x 10⁷/ml) of SMR CAR bacillus suspension were used as positive controls. SPF non-infected control rat lungs were used as negative controls.

Lung samples for histopathology were fixed in 10% buffered formalin, routinely processed, embedded in paraffin-wax, sectioned at 3 µm and stained with hematoxyline-eosine (HE) and WS stains.

For immunohistochemical labelling, sections were de-waxed and immersed in H₂O₂ 3% in absolute methanol for 30 min. They were then rinsed in phosphate buffered saline (PBS) and placed in a solution of 0.1% protease, pH 7.2, for 5 min at room temperature. The sections were washed three times in PBS, blocked with 5% normal goat serum for 30 min and exposed to polyclonal antisera against rat CAR bacillus applied at dilutions ranging from 1:100 to 1:1200 for 2 hr. The sections were rinsed in PBS and incubated for 30 min with a 1:5 dilution of biotinylated antibody to mouse and rabbit Ig G (LSAB Kit; Dako, Burlingame, CA, U.S.A.), rinsed again in PBS, and incubated for 30 min with a 1:5 dilution of streptavidin-peroxidase complex reagent (LSAB Kit; Dako, Burlingame, CA, U.S.A.). All incubations were at room temperature. After rinsing in PBS the sections were exposed to 3-amino-9-ethylcarbazole (AEC) (Sigma, St Louis, MO, U.S.A.) for 5 min. Same procedure was carried out using 3,3'-diaminobenzidine (DAB) (Sigma) as substrate. They were then counterstained with Mayer's haematoxyline and mounted using an aqueous mounting medium (Immu-mount: Shandon, Pittsburgh, PA, U.S.A.).

*CORRESPONDENCE TO: Dr. Orós, J., Veterinary Faculty of Las Palmas, c/Fco. Inglett Artilles, 12A, Las Palmas de Gran Canaria, Canary Islands, Spain.
ABC staining was performed by using the same anti-rat
CAR bacillus sera applied at the same dilutions and ABC
staining kit (Vectastain ABC kit; Vector Labs, CA, U.S.A.)
using both AEC and DAB as chromogens. Negative
procedure controls consisted in the use of antisera against
other pathogens above described and the substitution of
primary antibody by normal goat serum or PBS.

Severe lesions were observed through the airways of
rats inoculated with both inocula of the CAR bacillus SMR
strain. Various degrees of bronchiectasis with mononuclear
cell infiltration and mononuclear cell follicles with germinal
centers around the bronchi were observed in the affected
lungs. In the affected bronchi and bronchioles, the epithelial
layer associated with an increase in number of goblet cells
was increased in height twice to three times the normal.
Numerous filamentous bacteria were detected by WS
method in these regions covering the epithelia, existing in
large quantities particularly in the rats inoculated with the
more concentrated inoculum.

Immunohistological results are summarized in Table 1.
Strong immunoperoxidase red labelling of bacteria lining
the ciliated bronchial epithelium from the CAR bacillus
infected rats was clearly detected using the anti-rat CAR
bacillus mouse sera at a dilution of 1:800 (Fig. 1) whereas
immunoreaction was not observed in the pulmonary ciliated
epithelium of non-infected rats when the same sera were
used. Immunoreactivity was exclusively confined to the
CAR bacilli lined amongst and parallel to cilia and was not
observed within the cytoplasm of epithelial cells.
Background was not evidenced at this dilution using AEC
as substrate. However, moderate background was detected
using DAB as substrate. No immunoreaction was detected
when *Salmonella* sp., *Mycoplasma pulmonis* and *Pasteurella*
sp. antisera were applied to the same preparations.
Comparative data concerning ABC method are shown in
Table 1.

The results of the present study indicate that this
immunohistochemical test using polyclonal antisera can be
used to detect CAR bacillus in pneunonic lesions from

![Fig. 1. Immunoperoxidase red labelling of rat CAR bacilli
lining the ciliated bronchial epithelium. Mayer's
haematoxyline counterstain. × 1,000.](image)

<table>
<thead>
<tr>
<th>Inoculation Dose</th>
<th>0.1 ml 8.5×10⁷</th>
<th>0.1 ml 7.5×10⁷</th>
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<tr>
<td>Method</td>
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<td>Dilution sera anti-CAR bacillus</td>
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<tr>
<td>Substrate</td>
<td>Specific IP&lt;sup&gt;a&lt;/sup&gt; staining</td>
<td>Non-specific background</td>
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<td>AEC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+++&lt;sup&gt;f&lt;/sup&gt;</td>
<td>+++&lt;sup&gt;g&lt;/sup&gt;</td>
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<td>DAB&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+++&lt;sup&gt;j&lt;/sup&gt;</td>
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<sup>a</sup> IP: immunoperoxidase. <sup>b</sup> LSAB: Labelled Streptavidin Biotin. <sup>c</sup> ABC: Avidin Biotin Complex. <sup>d</sup> AEC: 3-amino-9-ethylcarbazole. <sup>e</sup> DAB: 3,3'-diaminobenzidine. <sup>f</sup> +++: intense. <sup>g</sup> ++: moderate. <sup>h</sup> +: weak. <sup>i</sup> --: absent.
infected rats and to study the pathogenesis of this disease.

The specificity of antibody to rat CAR bacillus was confirmed by the fact that specific immunoreaction was not apparent in SPF non-infected normal rat lungs.

The pattern of immunostaining was consistent with previously described IF [14], being confined to the ciliated bronchial epithelium, and confirms the previously described distribution of CAR bacillus [3, 4, 8, 10, 15, 17, 18]. However, compared to the IF, this red immunoperoxidase labelling allowed to detect individual bacilli and immunofluorescence microscope was not required. On the other hand, the ABC method was less sensitive than the LSAB method as is described in previous studies with other bacteria [5].

The use of AEC as substrate of the immunoreaction was evaluated more efficient than DAB because of the background observed using DAB and the strong red colour provided by AEC facilitating the interpretation of results.

According to the results of the present study, it can be concluded that the immunoperoxidase technique based on LSAB method using AEC as substrate is a very useful method for the detection of CAR bacillus antigen in paraffin-embedded rat lungs.

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REFERENCES