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## Production and characterization of monoclonal antibodies against *Taylorella equigenitalis*

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**Summary** — Monoclonal antibodies were produced against *Taylorella equigenitalis* using two reference strains. Out of the 79 hybridoma clones shown to express antibodies to *T equigenitalis* by indirect immunofluorescence assay, 16 were selected for monoclonal antibody production and characterization. These clones recognized different field strains of *T equigenitalis* isolated in France. They showed no cross-reaction with bacterial strains with previously reported antigenic cross-reactivity, nor did they react with other bacteria commonly found in genital flora. The epitopes recognized by eight of the monoclonal antibodies were situated in proteins of 150, 120, 52.7 and 22 kDa. These epitopes were resistant to the extraction denaturing conditions. These monoclonal antibodies could be used as reagents for specific detection of *T equigenitalis*.

**monoclonal antibody / *Taylorella equigenitalis* / contagious equine metritis**

**Résumé** — **Production et caractérisation d'anticorps monoclonaux anti-*Taylorella equigenitalis*.** Des anticorps monoclonaux ont été produits contre deux souches de référence de *Taylorella equigenitalis*. Sur 79 clones d'hybridomes obtenus après screening par le test d'immunofluorescence indirecte, 16 ont été sélectionnés pour la production d'anticorps monoclonaux et caractérisés. Les anticorps monoclonaux reconnaissent différentes souches isolées en France. Ils ne reconnaissent pas les bactéries pour lesquelles des croisements antigéniques ont été rapportés, qui constituent habituellement la flore génitale. Ces anticorps monoclonaux étaient dirigés contre les protéines de 150, 120, 52,7 et 22 kDa. Les épitopes de ces protéines résistaient à la dénaturation par le 2-mercaptoéthanol. Ces anticorps monoclonaux pourraient servir de réactifs spécifiques pour la détection de *Taylorella equigenitalis*.

**anticorps monoclonal / *Taylorella equigenitalis* / métrite contagieuse équine**

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## INTRODUCTION

*Taylorella equigenitalis*, the causative agent of contagious equine metritis (CEM), was first described by Taylor et al (1978). Since the first outbreak of this disease in 1977 in Newmarket (United Kingdom), CEM has spread throughout the equine population of the world (Europe, USA, Japan) and many countries have since developed control programs based on the isolation of the bacteria.

CEM was initially characterized by outbreaks of purulent vaginal discharges caused by acute endometritis. The epidemiology and clinical manifestations of the disease have now changed, particularly in France. Clinical metritis outbreaks have become scarce and *T equigenitalis* has been mostly isolated from subclinical or asymptomatic carriers. As *T equigenitalis* is a very fragile and slow-growing bacteria (Guérin, 1992), its culture is not a reliable diagnostic method. Other diagnostic tools would be necessary for effective control programs, based on antigen or DNA recognition. Vaissaire and Tram (1992) developed an indirect immunofluorescence assay (IIF) which is more sensitive and rapid than bacteriology.

*T equigenitalis* cross-reactivity with other bacteria is controversial. It has been found by some authors (Smith and Young, 1978; Saxegaard, 1979; Neill et al, 1984; Ter Laak and Wagenaars, 1990) and not by others (Dabernat et al, 1980; Lapan et al, 1991; Vaissaire and Tram, 1992). All the above-mentioned studies have been performed with conventional polyclonal antisera using crude bacterial extracts. In order to analyse the antigenic cross-reactivity and to improve diagnostic methods that are based on antigen recognition, we undertook this study to develop monoclonal antibodies (mAbs) against *T equigenitalis*. In this paper, we exhibit preliminary results upon the specificity of our monoclonal antibodies against *T equigenitalis*.

## MATERIALS AND METHODS

### Bacterial strains

#### *T equigenitalis* strains

Nine strains of *T equigenitalis* were used in this study: two reference strains (R1-16 and R2-19) obtained from Dr Vaissaire (Centre national d'études vétérinaires et alimentaires, Laboratoire central de recherches vétérinaires, Maisons-Alfort, France) and seven field strains isolated in four districts situated in the north west of France (Indre-et-Loire, Calvados, Côtes-d'Armor and Orne) (table I).

All the strains were grown in chocolate agar plates with or without actidione and streptomycin (AES laboratoire, Combourg, France) and incubated in a 7% CO<sub>2</sub> humid atmosphere.

The enzymatic reaction and sugar fermentation analyses were carried out using the API-NH system (bioMérieux, Marcy-l'Étoile, France). Furthermore, the bacteria were tested for catalase, cytochrome oxidase activity and by serum agglutination test (SAT), using polyclonal

**Table I.** Strains of *T equigenitalis* used for preparation and characterization of monoclonal antibodies.

Strain designation/ original designation	Source	Streptomycin resistance
R1-16/16	CNEVA	S
R2-19/19	CNEVA	R
1/129S	LVD37	R
2/1	LVD14	R
3/12.397	LDA22	R
4/26.658	LDA22	R
5/7001-01	LDA22	R
6/250	LDO61	R
7/715	LDO61	R

S = sensitive; R = resistant.

rabbit antiserum (CNEVA-LCRV, Maisons-Alfort, France).

### **Other bacterial strains**

In order to verify the specificity of monoclonal antibodies, some bacterial strains were used, which are described as having cross-reactions with the *T equigenitalis* antisera or which are common in the genital flora: *Actinobacillus equuli*, *Pseudomonas aeruginosa*, *Pasteurella multocida*, *Pasteurella haemolytica*, *Streptococcus equi*, *Staphylococcus aureus*, *Pseudomonas fluorescens* and *Klebsiella pneumoniae*. These were cultivated on Columbia blood agar plates.

### **Preparation of bacterial suspension for immunization**

The R1-16 and R2-19 reference strains were washed twice in phosphate-buffered saline (PBS) 0.1 M pH 7.4 and inactivated by heating at 56 °C for 75 min. The cells were then diluted in PBS, until the optical density of the suspension was 0.77 when examined by spectrophotometer at 380 nm (Tainturier et al, 1981), and finally aliquoted and stored at -80 °C.

### **Mouse immunization and hybridoma production**

Adult BALB/C mice were injected intraperitoneally with 0.5 mL of the R1-16 and R2-19 bacterial suspensions emulsified with Freund's complete adjuvant (two mice/strain). A booster injection was given on day 14 with the same preparation. On day 21, the mice were immunized with 0.2 mL of suspension without adjuvant by the intravenous route and the spleen cells were collected 2 days later. The hybridoma cells were produced according to the standard procedure (Kohler and Milstein, 1975). Briefly, murine myeloma SP2/O-Ag14 cells and immune spleen cells were fused in a 1:5 ratio using PEG 1500 (Sigma, l'Isle-d'Abeau, France) and maintained

in 96-well tissue culture plates containing mouse macrophages or spleen feeder cells or OPI supplement (Sigma) in HAT-DMEM selective medium. The hybridomas were tested by IIF and the positive ones were transferred for expansion into 24-well plates containing HT-DMEM medium prior to cloning. After 4-7 days, the hybridomas were cloned by the limiting dilution method to obtain a single cell per well in a 96-well tissue culture plate using HT-DMEM medium and splenic cells as feeders. The wells that contained a single clone were screened by IIF and the positive cells were frozen in liquid nitrogen. Some hybridomas were further propagated for monoclonal antibody production and characterization. Hybridoma tissue culture supernatants were buffered by addition of 1:20 volume of 1 M Tris (pH 8.0) and sodium azide (0.02%), aliquoted and stored at -20 °C.

### **Screening of hybridomas by indirect immunofluorescence assay**

The standard IIF procedure described by Vaisaire and Tram (1992) was used. After two washings in PBS 0.1 M, pH 7.4, the bacterial strains were resuspended in the same PBS containing 1% formaldehyde in order to obtain a suspension with a turbidity of one on the McFarland scale. Thirty microliters of this suspension was applied on each spot of fluorescent slides. After drying (15 min at 37 °C), the slides were fixed in pure acetone for 15 min at room temperature. After drying, the slides were incubated with 40 µL of hybridoma supernatants (30 min at 37 °C). The slides were washed in a PBS bath under agitation for 15 min. After rinsing in distilled water and drying, the slides were incubated 30 min at 37 °C with 40 µL of a solution of fluorescein isothiocyanate conjugate rabbit anti-mouse F(ab)2 fraction (Eurobio, Les Ulis, France) diluted at 1:40 in PBS with blue Evans (1:10 000). Finally, the slides were washed in PBS, rinsed in distilled water, dried as above, mounted in glycerin with 1% PBS and examined with a fluorescent microscope. A serum from nonimmunized mouse was used as a negative control. The FITC antiserum conjugate

was also incubated with all the bacterial strains for conjugate control.

### **Preparation of *T equigenitalis* protein extracts**

#### ***Nondenatured extract of T equigenitalis (NE)***

The *T equigenitalis* cells were collected by centrifugation (6 000 g, 10 min) and washed three times in PBS 0.1 M, pH 7.4. The pellets were resuspended in a small volume of SDS buffer (2% sodium dodecylsulfate (SDS), PBS pH 7.4) and incubated at 37 °C for 30 min. After this process, proteins keep their biological activity (Bhunja et al, 1991; Gabriel and Gesten, 1992). After SDS buffer extraction, the cells were checked under phase contrast microscope for cell lysis. After centrifugation (10 000 g, 10 min) the supernatants containing NE were dialyzed exhaustively against deionized water at 4 °C for 48 h, aliquoted and stored frozen (-80 °C) for future use. The protein concentration of NE was determined using the Bio-Rad Protein Assay (BioRad, Ivry-sur-Seine, France).

#### ***Lipopolysaccharide (LPS) extract***

Proteinase K-digested NE were used as the LPS extracts (Hanner et al, 1991). A 10 µL portion of the NE was diluted with 35 µL of LPS digestion buffer: 0.0625 M Tris-HCl, pH 6.8; 0.1% SDS; 10% glycerol and 5 µg of proteinase K (Sigma). These preparations were incubated at 37 °C for 1 h and heated to 100 °C for 5 min prior to electrophoresis.

#### **Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)**

The NE of *T equigenitalis* strains were dissolved in a sample solvent (0.1 M Tris-HCl pH 6.8; 10% glycerol; 2% SDS; 2 mM β-mercaptoethanol and 0.01% bromophenol blue) to obtain a concentration of 1 mg of protein/mL and boiled at 100 °C for 5 min (denatured extract

of *T equigenitalis*, DE). For the separation of the bacterial proteins, a discontinuous SDS-PAGE was used (Laemmli, 1970). The separating gel was made from 12% acrylamide and the stacking gel from 4% acrylamide. Twenty microliters of each DE sample was loaded into the bottom of the slots at a concentration equivalent to 5 µg of protein per lane. The electrophoresis was performed at 100 V, 50 mA (constant current) for 10 h in a vertical slab gel unit (Hoefer Scientific Instr, San Francisco, CA). For the molecular weight determinations, the low molecular weight calibration kit (Pharmacia-Biotech, Saint-Quentin-en-Yvelines, France) was used. For the visualization of the bands in the polyacrylamide matrice, we used Coomassie R350 staining (Pharmacia-Biotech, France) and for the LPS components, silver staining (Tsai and Frasch 1982).

#### **Immunoblotting**

The protein bands were transferred from the gel onto an immobilon PVDF membrane (Millipore Corp, Saint-Quentin-en-Yvelines, France) by electroblotting using a MiniTrans-Blot electrophoretic transfer cell (Bio-Rad) with a transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3) at 100 V, 250 mA for 1 h. In order to verify the electrophoretic transfer conditions and to identify the protein bands on the membranes, we used the Colloidal Gold Total Protein Stain (BioRad). After the transfer, the membranes were submerged for 30 min in a blocking solution (3% gelatin in 20 mM Tris and 0.5 M NaCl) and rinsed by gentle agitation in wash solution (20 mM Tris, 0.5 M NaCl, 0.05% Tween 20). The membranes were then exposed to dilutions of mAbs diluted from 1:100 to 1:1 000 in antibody buffer (20 mM Tris, 0.5 M NaCl, 0.05% Tween 20, 1% gelatin) for 180 min at 25 °C. The binding of mAbs to the peptide bands was visualized with a goat anti-mouse IgG (H + L) alkaline phosphatase (AP) conjugate (Bio-Rad) (1:2 000 dilution) and an AP substrate solution (Bio-Rad). A positive serum from mice immunized with *T equigenitalis* reference strain

and a negative serum from a nonimmunized mouse was used to check the procedure.

### Dot-blotting

Immobilon PVDF membranes (Sigma) were prewetted with a solution of methanol (100%) for 1–3 s, immersed in distilled water for 1–2 min to elute methanol and equilibrated in washing solution (20 mM Tris, 500 mM NaCl, 0.05% Tween 20, pH 7.5). The NE and DE were bound to the membranes during a 1 h incubation at room temperature. The dot membranes were washed twice for 10 min in washing solution and then submerged in blocking solution (3% gelatin in 20 mM Tris and 500 mM NaCl) for 1 h. The membranes were washed twice as above and incubated with selected mAbs in the same conditions as for the immunoblotting. The binding of the mAbs to the dot blot membranes was revealed with a goat antimouse IgG (H + L) AP conjugate and AP substrate solution (Bio-Rad). The same positive control serum as in immunoblotting was used together with a negative serum from a nonimmunized mouse.

### Isotype determination

For the determination of the mouse monoclonal antibody isotype, we used the Sigma immunotype kit which consists of nitrocellulose pre-coated strips that specifically capture the mouse immunoglobulin. After further incubations, the identity of the mouse immunoglobulin isotype is revealed by a biotin–avidin–enzyme detection system.

## RESULTS

### Bacterial strains

After Gram staining, the strains of *T equigenitalis* cultivated on agar chocolate chiefly exhibited a Gram-negative cocobacillary form. The analysis of the biochemical characteristics of *T*

*equigenitalis* revealed that all strains were catalase and cytochrome oxidase positive. The API-NH system analysis revealed that all strains were alkaline phosphatase and gamma glutamyl transferase positive (except field strain 5, negative in gamma glutamyl transferase), penicillinase, ornithin-decarboxylase, urease, lipase, beta-galactosidase and proline-aminase negative. No sugar (glucose, fructose, maltose and saccharose) was metabolized by any of the *T equigenitalis* strains. In SAT, all the strains were agglutinated by polyclonal rabbit antiserum. In SDS-PAGE, all the *T equigenitalis* strains produced essentially similar protein migration patterns (fig 1). The electrophoresis revealed that each of the *T equigenitalis* isolates had a complex protein profile with over 35 bands as well as LPS. The patterns of the LPS components were detected after silver staining and were relatively uniform for all the *T equigenitalis* strains with a major component having an apparent molecular mass of approximately 22 kDa.

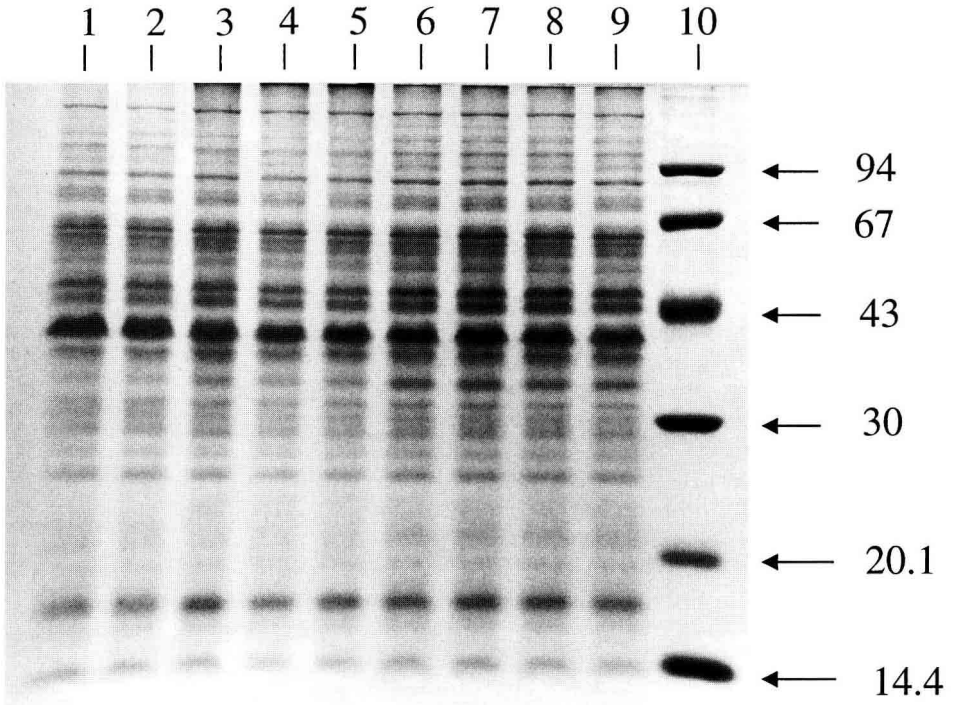
### Hybridomas

After two fusions, hybridomas were generated in approximately 820 out of a total of 1 020 wells (81.37%). Only 60 wells were tested in IIF for the detection of hybridomas that produced anti-*T equigenitalis* antibodies and 35 (58%) were positive. The hybridomas from nine positive wells were cloned by the limiting dilution method (3, 1 and 0.3 cells/well) using spleen feeder cells. A total of 161 single clones was obtained and these were tested by IIF. The 79 positive clones were frozen in liquid nitrogen. Among the 79 clones, 16 were further propagated for antibody production and characterization.

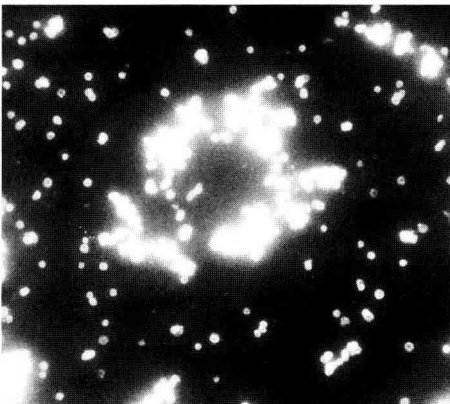
### Characterization of monoclonal antibodies

#### *Specificity of mAbs for T equigenitalis strains using IIF*

The 16 selected clones were tested by IIF (fig 2) with the seven field strains of *T equigenitalis*



**Fig 1.** SDS-PAGE profile of nine strains of *T. equigenitalis*. From left to right, lanes contain preparations of the following strains: lane 1 strain 1 (129S/LVD37); lane 2 strain 2 (1/LVD14); lane 3 strain 3 (12.397/LDA22); lane 4 strain 4 (26.658/LDA22); lane 5 strain 5 (7001-01/LDA22); lane 6 strain 6 (250/LVD61); lane 7 strain 7 (715/LVD61); lane 8 reference strain R-16 (16/CNEVA); lane 9 reference strain R-19 (19/CNEVA); lane 10 low molecular weight SDS-PAGE standards (Pharmacia-Biotech: 94 000 phosphorylase B; 67 000 albumin; 43 000 ovalbumin; 30 000 carbonic anhydrase; 20 100 trypsin inhibitor; 14 400 alpha-lactalbumin).



and they all reacted positively. Four mAbs (7B7.1; 7B7.7; 7B7.10; 10C9.6) showed a weaker response for some strains (table II).

In order to verify the specificity of *T. equigenitalis* mAbs, some bacterial strains were selected that had been reported to show cross-reactions with *T. equigenitalis* or which were common in the genital flora. The 16 mAbs

**Fig 2.** IF assay (R19 reference strain) using mAbs anti-*T. equigenitalis* x 100 immersion. The cell wall is green with a non-fluorescent center.

**Table II.** The specificity of 16 monoclonal antibodies anti-*T. equigenitalis* using indirect immunofluorescence assay.

<i>mAbs designation</i>	<i>R-1</i>	<i>R2-16</i>	<i>R2-19</i>	1	2	3	4	5	6	7	A equuli	P aeruginosa	P multocida	P haemolytica	S equi	S aureus	S fluorescens	P pneumoniae	K
1 3B6.1	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
2 3B6.4	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
3 3B6.11	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
4 7B7.1	+	+	+	+	(+)	+	+	+	+	+	-	-	-	-	-	-	-	-	-
5 7B7.7	+	+	+	+	+	(+)	(+)	+	+	+	-	-	-	-	-	-	-	-	-
6 7B7.10	+	+	+	+	+	+	(+)	+	+	+	-	-	-	-	-	-	-	-	-
7 7B8.1	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
8 7C4.10	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
9 7D7	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
10 7D7.12	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
11 7D7.16	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
12 10C4.17	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
13 10C9.6	+	+	+	+	+	+	(+)	+	+	+	-	-	-	-	-	-	-	-	-
14 11C9.1	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
15 11C9.4	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
16 11C9.5	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-

+ Positive; (+) weak positive; - negative.

tested in IIF with these bacteria were all negative (table II).

#### *mAbs reactivity to T equigenitalis in SAT*

To test the mAbs reactivity in SAT, we only used the R-19 reference strain. Fourteen out of the 16 mAbs were positive (table III).

#### *Localization of specific epitopes*

To analyze the localization of the specific epitopes recognized by the selected mAbs, we compared the results of the immunoblottings with positive mouse serum and with the mAbs (fig 3). The positive sera collected from immunized mice, reacted with five proteins of the R-19 strain: 120, 52.7, 33.4, 17.5 and 22 (LPS) kDa. In immunoblotting, nine out of the 16 selected mAbs reacted positively and seven were negative. The specific epitopes for the positive immunoblotting reacting mAbs were situated in proteins of 150 kDa (3B6.1), 120 kDa (11C9.1), 52.7 kDa (7B8.1 and 7C4.10) and 22 kDa (7B7.10, 7D7.3, 11C9.4, 11C9.5) (table III). The

7B7.7 reacted positively in immunoblotting with two proteins (34.4 and 40 kDa) (fig 3).

#### *Reactivity of mAbs with T equigenitalis using dot-blotting*

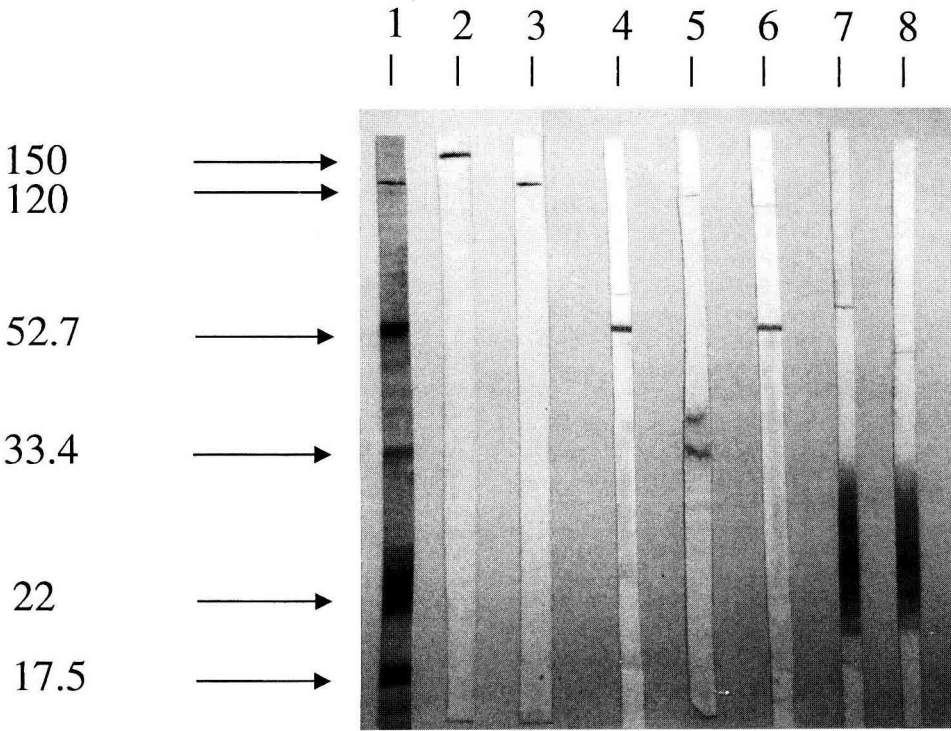
In order to investigate whether the negative results in immunoblotting were caused by epitopes that were destroyed by the denaturing reagents used in the preparation of the sample, the mAbs in dot blotting were tested with the denatured (DE) or the nondenatured (NE) extracts of the R-19 reference strain. Immunoblotting and dot blotting with the denatured antigens gave similar results except with the 7D7.12 clone, which was positive in dot blot and negative in immunoblotting (table III). Nondenatured antigens were however recognized by all the mAbs (fig 4).

#### *Isotypes*

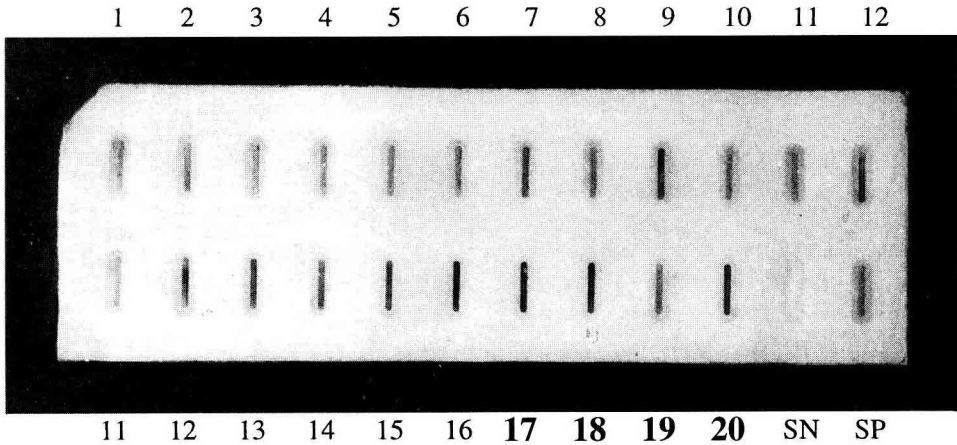
As shown in table III, we obtained 6 IgM, 4 IgG2b, 3 IgG3 and 2 IgG1 mAbs. The 7B7.7 'mAb' seemed to be a mixture of two mAbs,

**Table III.** Characterization of 16 monoclonal antibodies to the *T equigenitalis* R-19 reference strain.

	<i>mAbs designation</i>	<i>IIF</i>	<i>SAT</i>	<i>Immunoblot</i>	<i>Dot blot with denaturation</i>	<i>Dot blot without denaturation</i>	<i>Monoclonal specificity (kDa)</i>	<i>Isotype</i>
1	3B6.1	+	+	+	+	+	150	IgM
2	3B6.4	+	+	-	-	+		IgM
3	3B6.11	+	+	-	-	+		IgM
4	7B7.1	+	-	-	-	+		IgG1
5	7B7.7	+	-	+	+	+	34.4 + 40	IgG1 + IgG3
6	7B7.10	+	+	+	+	+	22 (LPS)	IgG1
7	7B8.1	+	+	+	+	+	52.7	IgG3
8	7C4.10	+	+	+	+	+	52.7	IgG3
9	7D7.3	+	+	+	+	+	22 (LPS)	IgM
10	7D7.12	+	+	-	+	+		IgM
11	7D7.16	+	+	-	-	+		IgM
12	10C4.17	+	+	-	-	+		IgG3
13	10C9.6	+	+	-	-	+		IgG2b
14	11C9.1	+	+	+	+	+	120	IgG2b
15	11C9.4	+	+	+	+	+	22 (LPS)	IgG2b
16	11C9.5	+	+	+	+	+	22 (LPS)	IgG2b



**Fig 3.** Localization of specific protein bands recognized by mouse positive serum and by mAbs in immunoblotting. lane 1 mouse positive serum; lane 2 mAb 3B6.1 (150 kDa); lane 3 mAb 11C9.1 (120 kDa); lane 4 mAb 7B8.1 (52.7 kDa); lane 5 mAb 7B7.7 (33.4 + 40 kDa); lane 6 mAb 7C4.10 (52.7 kDa); lane 7 mAb 7B7.10 (22 kDa) LPS; lane 8 mAb 7D7.3 (22 kDa) LPS.



**Fig 4.** Dot-blotting: lanes 1–16: mAbs anti-nondenatured R-19 reference strain (mAbs nos 1–16, table III); lanes 17–20: other mAbs anti-nondenatured R-19 reference strain (data not shown); SN: normal mouse serum; SP: positive serum from immunized mice.

each with its own isotype (Ig G1 and Ig G3) and its own epitope. The 7B7.7 hybridoma cells were obtained from a well with two physically separate clones.

## DISCUSSION

The laboratory diagnosis of CEM is based on the isolation of *T equigenitalis* by bacteriological culture from swabs and the identification of the agent by morphological and biochemical criteria. Because of the considerable fragility of the bacterium, its slow-growing character and its growth inhibition caused by other contaminating bacteria, bacteriological culture is an unreliable diagnostic. For these reasons, other tools based on antigens or DNA recognition seem to be required in order to perform a control program for the disease. The bacterium can be identified using direct or, preferably, indirect immunofluorescence which is a sensitive and rapid method (Vaissaire and Tram, 1992). In this case, highly specific antisera need to be used in order to eliminate false-positive reactions. For accurate antigen detection, there are two essential difficulties: the antigenic cross-reactivity of the agent, which is disputed by some authors, and especially, the heterogenic spectra of antibody reactivity of the polyclonal antisera, which may contain antibodies to a wide range of bacteria other than *T equigenitalis*. These problems can be easily solved with monoclonal antibodies.

We produced 79 hybridoma clones that synthesized monoclonal antibodies against *T equigenitalis* antigens, by indirect immunofluorescence screening of culture supernatants. Among these clones, only 16 were further propagated for mAb production and characterization.

For the characterization of the selected mAbs, we directed our investigations in three directions: a) the ability to recognize different strains of *T equigenitalis*; b) the reactivity to bacteria different from *T equigenitalis* but which, as mentioned by some authors, showed cross-reactivity with *T equigenitalis*; and c) the localization of the epitopes.

We verified whether the 16 selected mAbs may recognize different *T equigenitalis* strains (seven field strains isolated in France). All these mAbs reacted positively in IIF with the seven field strains and two reference strains of *T equigenitalis*. Despite the fact that the electrophoretic profile of all the studied strains was similar, which confirmed previous observations (Brewer and Corbel, 1983; Lapan et al, 1991) and that all studied strains were recognized by our mAbs, testing them against a wider range of *T equigenitalis* will be necessary in future works.

The specificity of the mAbs was confirmed by the fact that they failed to react in IIF with some bacterial strains that have been reported to show cross-reactivity with *T equigenitalis* – *A equuli* (Neill et al, 1984), *P multocida*, *P haemolytica* (Ter Laak and Wagenaars, 1990), *S aureus*, *P fluorescens* (Vaissaire and Tram, 1992) –, or other strains which are common in the genital flora, *S equi*, *K pneumoniae* and *P aeruginosa*. Thus, the positive reactions of the *T equigenitalis* polyclonal rabbit anti-sera frequently observed in IIF with *S aureus* and *P fluorescens* (Vaissaire and Tram, 1992) were not observed with our mAbs.

To analyze the localization of specific epitopes recognized by selected mAbs, we compared the results of immunoblots with mouse serum and with mAbs. The serum collected from immunized mice reacted with five proteins of the R-19 strain: 120, 52.7, 33.4, 17.5 and 22 (LPS) kDa. The specific epitopes for positive immunoblot reacting mAbs were situated in proteins of 150, 120, 52.7 and 22 (LPS) kDa. In order to find an explanation for the negative results obtained with seven out of 15 mAbs in immunoblotting, we performed dot-blot tests using nondenatured (NE) and denatured (DE) preparations of the R-19 reference strain. The seven mAbs that were negative in immunoblotting, were also negative in dot-blotting with the denatured preparations but they were positive with nondenatured ones. In nondenaturing conditions (treatment with SDS only) both the conformation and biological activity of most of the proteins remain intact (Gabriel and Gersten,

1992) but under reducing conditions (treatment with  $\beta$ -mercaptoethanol and high temperature) there were some conformational changes and epitope destruction. We therefore speculate that the loss in reactivity of some mAbs after denaturing extraction was due to conformational changes or epitope destruction. We did however produce eight mAbs which maintained their reactivity on the denaturing extracts. These eight mAbs could therefore be valuable reagents for the detection of *T equigenitalis* antigens.

In conclusion, using mAbs, we did not find the cross-reactivity between *T equigenitalis* and some other bacteria that has been reported by some authors to show cross-reactivity or bacteria which are common in genital flora (Smith and Young, 1978; Saxegaard, 1979; Neill et al, 1984). Our mAbs did not detect any antigenic difference between various strains of *T equigenitalis*. The monoclonal antibodies directed against some epitopes that are resistant to denaturing conditions could be employed as appropriate reagents for CEM diagnosis for methods based on antigen recognition. A future article will address the testing of our mAbs in the indirect immunofluorescence assay for CEM diagnosis.

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