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Short communication

Corynebacterium pseudotuberculosis : biochemical properties, production of toxin and virulence of ovine and caprine strains

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Summary — Twenty-two strains of *Corynebacterium pseudotuberculosis,* the causative agent of caseous lymphadenitis, were isolated from typical abscesses in sheep and goats from flocks in 6 different regions of France and were characterized. These strains were uniform in biochemical characteristics, susceptibility to 8 antimicrobial agents, and virulence for OF1 mice. All these strains produced an exotoxin, identified by inhibition of staphylococcal β-hemolysin. Moreover, this study showed that strain 19R, a streptomycin-resistant mutant used in experimental infection in sheep, possessed the same characteristics as the field strains of *C. pseudotuberculosis,* except for its resistance to streptomycin (500 μg/ml)

***Corynebacterium pseudotuberculosis* - ovine - caprine - bacteriology**

Résumé — Propriétés biochimiques, production de toxine et virulence de souches ovines et caprines de *Corynebacterium pseudotuberculosis.* *Vingt-deux souches de* Corynebacterium pseudotuberculosis, *agent causal de la lymphadénite caséeuse, isolées à partir d'abcès typiques chez des moutons ou chèvres appartenant à des troupeaux répartis dans 6 régions françaises différentes ont été caractérisées. L'ensemble de ces souches possède un profil biochimique (fermentation de 20 sucres, réduction des nitrates) et une sensibilité aux antibiotiques uniformes, sécrète une toxine et montre une virulence comparable pour la souris OF1. En outre, cette étude a permis de montrer que la souche 19R, sélectionnée pour une résistance à la streptomycine (500 μg/ml), possède les mêmes caractéristiques que la souche parentale à l'exception de la résistance à la streptomycine, permettant de la différencier des souches sauvages sensibles à des concentrations de streptomycine supérieures à 64 μg/ml.*

Corynebacterium pseudotuberculosis - *ovin - caprin - bactériologie*

Corynebacterium pseudotuberculosis is the causative agent of caseous lymphadenitis, a suppurative infection of lymph nodes and lungs in sheep and goats (Brown and Olander, 1987). Experimental infection of lambs or ewes need a representative strain that is easily distinguishable from field strains. The present study reports a comparative analysis of biochemical properties and virulence of several field strains and strain 19R, a streptomycin-resistant mutant used in experimental infection.

Eighty-seven bacterial strains, including 2 reference strains (CIP 59.46, Institut Pasteur, Paris, France and ATCC

19410, Rockville, USA), were used in this study, but only 22 strains, including strains 19 and 19R, were studied in detail. They were isolated from typical abscesses of sheep and goats from flocks in 6 different regions of France, except for reference strains. Strain 19 is a field strain isolated from an abscess of the prescapular lymph node in an adult ewe; strain 19R is a one-step streptomycin-resistant strain derived from strain 19 and obtained as previously described (Pierce-Chase *et al.,* 1964; Pardon and Marly, 1979). These strains, previously identified as *C. pseudotuberculosis* by other laboratories or by the authors with the common identification tests used for this bacterium (Muckle and Gyles, 1982; Collins and Cummins, 1986), were stored in a nutrient broth at –20°C before use. For characterization, strains were thawed and cultured twice on Trypticase Soy Agar (Biomérieux, Marcy l'Etoile, France), with 8% calf serum (TSAS) added. The 87 strains were examined for colony morphology and hemolysis after growth for 72 h on TSAS and sheep blood agar plates at 37°C. All strains were also tested for their ability (i) to produce a phospholipase D by inhibition of the cooperative hemolysis induced by the β-hemolysin of *Staphylococcus aureus* and a protein from *Streptococcus agalactiae* (strains 110 and 111 kindly supplied by Dr. Poutrel, Nouzilly, France) as described by Barksdale *et al.* (1981); (ii) to grow on TSAS to which 40 µg/ml of nalidixic acid had been added (Serva, Heidelberg, RFA); and (iii) to reduce nitrates to nitrites.

The fermentation reactions of 22 selected strains were tested for 20 sugars by inoculation of fermentation tubes containing 0.01% Phenol red with 50 µl of a homogenized bacterial suspension. Three to 4 drops of sterile calf serum were added to each tube; after 72 h of incubation tubes were recorded as positive when a yellow coloration was obtained, and variable when an orange coloration was obtained. The *in vitro* susceptibility of the 22 selected strains to 8 common antimicrobial agents was studied by determination of minimal inhibitory concentration (MIC; Washington and Slutter, 1980) by the dilution susceptibility test in solid TSAS. The virulence for OF1 mice (IFFA CREDO, L'Arbresle, France) of each of the 22 selected strains was tested by enumeration of viable bacteria recovered in spleen and liver on the sixth day following intravenous inoculation of 1.2 - 2.4 x 10^4 *Corynebacteria,* as previously described (Pardon and Marly, 1979; Pépin *et al.,* 1987). Three experiments (8 strains per experiment, 8 mice per strain) were performed and strain 19 was included in each experiment. Viable counts of *C. pseudotuberculosis* were expressed as $\log_{10}$ units. Means and standard deviations were calculated from the $\log_{10}$ values. The conventional F test was used for statistical analysis of results. For fermentation reactions, tests of virulence and susceptibility to antibiotics, bacterial suspensions were homogenized by sonication and standardized by turbidimetry at 600 nm (Pépin *et al.,* 1988).

Colonies of the 87 tested strains were surrounded by a narrow zone of hemolysis following the 72 h growth on sheep blood agar. All these strains were able to grow on TSAS to which nalidixic acid had been added, did not reduce nitrates to nitrites, and produced a substance which inhibited hemolysis by β-lysin of *Staphylococcus aureus.* Amylose, fructose, glucose, glycogen, maltose, mannose and soluble starch were fermented by the 22 selected strains. Arabinose, dulcitol, esculin, inositol, inulin, lactose, mannitol, sucrose, salicin and xylose were not fermented by any strain, while galactose, glycerol and trehalose fermentations were variable. The patterns of susceptibility to 8

common chemotherapeutic agents showed that there was a weak variability between the tested strains, including strain 19, except for strain 19R which was resistant to streptomycin with a MIC >256 µg/ml (Table I). No significant differences in virulence for OF1 mice measured by *in vivo* growth in spleen and liver between the 22 tested strains were found, despite a great variability (Table II).

The strains of *C. pseudotuberculosis* tested in this report and isolated in sheep or goats from various areas of France are uniform in their biochemical properties, production of exotoxin and susceptibility to antimicrobial agents. All tested strains grow on TSAS with nalidixic acid thus confirming that this selective agent can be commonly used for primary isolation of *C. pseudotuberculosis,* thus allowing inhibition of the main Gram-negative bacteria. The 87 tested strains do not reduce nitrates to nitrites: this biochemical pattern seems to be a constant characteristic of ovine or caprine *C. pseudotuberculosis* isolates, as described by numerous authors (Biberstein *et al.,* 1971; Muckle and Gyles, 1982; Barakat *et al.,* 1984; Songer *et al.,* 1988) with a few exceptions (Carne, 1940). All these strains produce a factor which inhibits staphycoccal β-hemolysin: this factor has been often identified as exotoxin of *C. pseudotuberculosis* by previous workers (Lovell and Zaki, 1966; Onon, 1979; Barksdale *et al.,* 1981; Hsu *et al.,* 1985). However, the relationship between the different exotoxic activities of *C. pseudotuberculosis* remain to be elucidated (Muckle and Gyles, 1986). The uniform fermentation reactions and susceptibility to 8 antimicrobial agents of 22 selected strains has been previously reported (Muckle and Gyles, 1982). All these characteristics of *C. pseudotuberculosis* confirm results of studies carried out in other countries (Carne, 1940; Domenech, 1980; Muckle and Gyles, 1982; Garg *et al.,* 1985; Songer *et al.,* 1988). In addition, in this report, the murine model provides evidence that respective virulence of various field strains of *C. pseudotuberculosis* is com-

Table I. Ranges of minimal inhibitory concentration values of strain 19R and 21 field strains of *Corynebacterium pseudotuberculosis.*

	Minimal inhibitory concentration values (µg/ml) [a]	
Antimicrobial	*Field strains*	*Strain 19R*
Ampicillin	0.25	0.25
Chloramphenicol	0.125–8	0.125
Erythromycin	≤ 0.125	≤ 0.125
Nalidixic acid	128–256	256
Neomycin	8–32	16
Penicillin G	0.125–0.25	0.125
Streptomycin	16–64	>256
Tetracycline	2	2

[a]Plates were read after 72 h of incubation at 37 °C.

Table II. Origin and virulence for OF1 mice of 22 strains of *Corynebacterium pseudotuberculosis.*

Exp. strain No.	*Origin*		*Inoculum (x10 $^{-4}$)*	*Log_{10} viable bacteria (m ± SD)*	
	Species	*Region*[a]		*Spleen*	*Liver*
Experiment 1					
19	sheep	1	1.3	5.65 ± 0.66	6.39 ± 0.57
19R	sheep	1	1.6	4.47 ± 0.85	5.97 ± 0.86
CIP 59.46	sheep	ref.	1.5	6.17 ± 1.21	6.95 ± 0.83
8	sheep	3	1.5	4.74 ± 0.82	6.16 ± 1.03
18	sheep	1	1.4	4.69 ± 1.19	6.08 ± 1.34
49	sheep	4	1.3	4.23 ± 1.24	5.64 ± 1.28
62	sheep	2	1.3	3.51 ± 0.76	5.24 ± 0.73
72	goat	2	1.2	4.56 ± 1.13	6.16 ± 0.99
76	sheep	3	2.0	4.80 ± 1.09	5.80 ± 1.31
Experiment 2					
19	sheep	1	1.6	4.74 ± 1.04	5.61 ± 1.45
9	sheep	1	1.9	4.00 ± 0.76	4.98 ± 0.87
85	sheep	1	2.0	4.84 ± 1.32	5.72 ± 1.38
105	goat	2	1.5	3.38 ± 0.83	5.33 ± 1.47
111	goat	6	1.6	4.55 ± 1.77	6.47 ± 1.54
138	sheep	1	1.7	5.18 ± 1.28	6.02 ± 1.47
147	sheep	1	1.7	5.72 ± 1.45	6.33 ± 1.32
153	goat	5	1.6	4.26 ± 1.30	5.52 ± 1.50
Experiment 3					
19	sheep	1	1.5	5.14 ± 1.40	6.78 ± 0.64
ATCC 19410	sheep	ref.	1.2	3.56 ± 0.92	4.92 ± 1.88
166	sheep	1	1.2	4.32 ± 0.76	6.19 ± 0.87
179	sheep	1	1.3	4.82 ± 1.17	6.56 ± 1.36
181	goat	5	1.6	4.76 ± 1.23	6.22 ± 1.28
182	goat	1	1.5	4.17 ± 0.80	5.22 ± 1.13
187	goat	1	1.5	4.25 ± 1.12	5.35 ± 1.38

[a] 1, INRA-Nouzilly (37); 2, INRA-Brouessy (78); 3, Rhône-Alpes (69, 38, 05); 4, Aveyron (12); 5, Station régionale de pathologie caprine - Niort (79) ; 6, INRA-Lusignan (86); ref., reference strain.

parable. Moreover, strain 19R possesses similar virulence characteristics and similar biochemical properties to field strains, except for its resistance to streptomycin; this confirms that 19R is a representative strain of *C. pseudotuberculosis* and it can be easily distinguished from various field isolates and its parental strain. Strain 19R is a reliable strain for experimental infection, allowing distinction between natural and experimental infection (Pépin *et al.*, 1988).

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