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Characterization of *Mycoplasma pulmonis* Variants Isolated from Rabbits

II. Basis for Differentiation of Antigenic Subtypes[1]

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The antigen composition of *Mycoplasma pulmonis* variants was studied by complement-fixation, agar-gel diffusion, and growth-inhibition tests. Two classes of complement-fixing antigens were demonstrated for *M. pulmonis* strains 47 and 63: (i) cross-related, heat-labile, water-soluble antigens, and (ii) high-titered, subtype-specific, heat-stable, water-soluble antigens. Lipid antigens prepared by organic solvent fractionation were low-titered antigens and showed little specificity. With the aid of agar-gel double-diffusion plates, the subtype-specific antigens were found to be precipitated by trichloroacetic acid and to be stable to periodate, but they were inactivated by pronase. Pronase-stable, periodate-labile precipitating antigens were observed as common components between the two variants. Antisera prepared with boiled antigens were found to be serologically active on gel diffusion but lacked neutralizing ability in growth-inhibition tests. Each of three strains of *M. pulmonis* (47, 63, ATCC 14267) could be identified as a variant because each strain possessed immunologically distinct heat-stable subtype-specific antigen(s).

Two strains of *Mycoplasma* isolated from rabbits have been shown to be related to each other and to *M. pulmonis* ATCC 14267 by growth inhibition and agar-gel double-diffusion tests (3). Although these three strains shared antigenic components, each could be distinguished on gel diffusion by the demonstration of one or more specific antigens.

The nature of the specific antigens of the two rabbit *Mycoplasma* strains, 47 and 63, was investigated by simple chemical and physical characterization. The most striking findings of the study were: (i) the major complement-fixing (CF) antigen of *M. pulmonis* was found to be a heat-stable "protein," and (ii) *M. pulmonis* species could be subtyped with this antigen.

MATERIALS AND METHODS

Mycoplasma. Included in this study were *M. pulmonis* (strains 47 and 63) described in the preceding paper (3) and *M. pulmonis* ATCC 14267 obtained from the American Type Culture Collection.

Mycoplasma concentrates and antisera. The methods of cultivating *M. pulmonis* and of preparing concentrated *Mycoplasma* suspensions and antisera were previously described (3). "Boiled" antigens were concentrates heated at 100 C for 30 min.

Fractionation of 63 and 47. Lipid extracts of "raw" antigens were prepared as described by Kenny and Grayston (7). [The term lipid refers to compounds soluble in chloroform-methanol but not removed when that phase is partitioned with 0.1 M KCl (5).] Suspensions were shaken vigorously with 100 ml of chloroform and 50 ml of methanol; 37.5 ml of 0.1 M aqueous KCl were added, and the mixture was shaken again. The chloroform and water-methanol phases were separated and the water-methanol phase was extracted twice more with chloroform. The chloroform phase was allowed to evaporate, the residue was resuspended in alcohol to the original volume of concentrate, and the product was termed *lipid (L) antigen.*

Trichloroacetic acid was added to the delipidized water-methanol phase to a concentration of 20% after removing methanol partially by evaporation, or totally by dialysis against water. The mixture was allowed to precipitate in an ice bath. The precipitate was removed by centrifugation at 650 × *g* for 10 min and was resuspended in a volume of 0.85% NaCl, equal to or half that of the "raw" antigen concentrate. Both the trichloroacetic acid-precipitated fraction and the supernatant fraction were dialyzed at 4 C for 2 days in distilled water and then for 1 day in 0.85% NaCl. The trichloroacetic acid precipitate, resuspended and dialyzed, was termed the *protein fraction.*

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The dialyzed supernatant phase was evaporated to the original volume of "raw" antigen and the aqueous residue was referred to as the *polysaccharide* fraction.

Periodate oxidation. A method of periodate treatment modified from that of Stacey and Barker (12) was performed on "raw" and boiled antigens as well as on the protein fraction of 63 and 47, to determine whether any of these preparations contained an antigenic polysaccharide component which would be inactivated by periodate. A saturated solution of sodium metaperiodate ($NaIO_4$) was prepared. (More than 0.1 M, but less than 1.0 M $NaIO_4$ could be dissolved in deionized water.) Antigens used in this treatment were in deionized water; oxidation was carried out in dark-brown bottles. Dilutions of antigen to periodate solution varied from 10:1 to 1:1. Time and temperature of periodate treatment varied from 5 to 24 hr at room temperature and 1 to 4 days at 4 C.

Pronase digestion. Strain 63 and 47 "raw" and boiled antigens and "protein" fractions were treated with pronase (Calbiochem, Los Angeles, Calif.) to discover whether any precipitating antigens could resist attack by this enzyme. Pronase is considered capable of catalyzing the hydrolysis of all peptide bonds and should destroy any protein antigen. A stock pronase solution was prepared at a concentration of 1,000 μg/ml in *p*H 7 phosphate buffer. The solution was incubated for 10 min at 37 C to destroy protein impurities. Antigens in phosphate-buffered saline (PBS) were treated with pronase at a concentration of 100 μg/ml, and these mixtures were incubated for 1 hr at 37 C.

CF test. The CF test employed was a microtiter technique (microtitration equipment obtained from Cooke Engineering Co., Alexandria, Va.) with overnight fixation at 4 C. Two full units of guinea pig complement (BBL) and two units of anti-sheep hemolysin were used. Block titrations of antigen and antisera were performed. End-point titers for each were considered to be the highest dilution capable of fixing complement so that hemolysis of sheep red blood cells could be completely prevented (4+ fixation).

Agar-gel double-diffusion tests. Immuno-Plates from Hyland Laboratories, Los Angeles, Calif., were used. Drawings of precipitation lines for each antigen-antibody system represent a summary of observations made over 1 week on plates with wells in agar spaced 1, 2, and 4 mm apart (Pattern A). Procedures for diffusion tests were described previously (3).

Growth-inhibition test. The procedure for this test was also described previously (3).

RESULTS

Classes of M. pulmonis CF antigens. The heat stability and organic solvent solubility of the antigens of *M. pulmonis* strains 47 and 63 were tested by comparing the CF antigen titers of "raw," boiled, and lipid extracts of the two strains with homologous and heterologous antisera. Results of these block titrations are summarized in Table 1, together with results of CF antigenic relationships with ATCC 14267. It was found that "raw" antigens boiled for as long as 30 min were as active as unheated "raw" antigens against homologous antiserum. However, the antigen titers of lipid extracts were low when measured by either homologous or heterologous antisera. Antigen titers of boiled concentrates were similarly low for heterologous antisera. Furthermore, heterologous antigen titers obtained with "raw" antigens were higher than those for lipid antigens (or boiled antigens) but were lower than those for the homologous "raw" antigens. Therefore, it was concluded that: (i) although two organisms were related, they could be distinguished by CF block titrations, and (ii) three classes of CF antigen could be described by

TABLE 1. *Comparison of antigen classes of Mycoplasma pulmonis strains 63, 47, and ATCC 14267 by complement-fixation*

Antigens	Antisera					
	Anti-63		Anti-47		Anti-14267	
	Ag[a]	Ab[b]	Ag	Ab	Ag	Ab
63 "raw"	1,024	256	128–256	256		
63 boiled	1,024	128–256	16	64	ND[c]	ND
63 "lipid"	16	256	4–8	64		
47 "raw"	128	256	1,024	512		
47 boiled	4–8	32	1,024	512	ND	ND
47 "lipid"	4	128	16	256		
14267 "raw"	16	256	16	256	32–64	256
14267 boiled	8	64	8	64	32–64	256

[a] Ag = antigen titer (highest dilution of antigen giving 4+ fixation in a block titration).
[b] Ab = antibody titer.
[c] ND = not done.

this method: (a) low-titered cross-related lipid antigens, (b) cross-related, heat-labile, water-soluble antigens, and (c) subtype (used synonomously with subspecies throughout) -specific, water-soluble, heat-stable antigens as the major CF antigen for each strain. These data agree with observations by others who used CF for serotyping *M. pulmonis* strains; e.g., whereas heterologous strains did appear to share CF antigens, antigen titers for homologous systems were at least one dilution higher than those for heterologous systems (4, 10).

The existence of low-level CF antibody to *M. pneumoniae* lipid antigen in unimmunized rabbits (6, 8) led to the suspicion that this antibody might have arisen from *M. pulmonis* infection. Further indications were that *M. pulmonis* might have a related lipid antigen. This hypothesis is unlikely because: (i) the antigen titers of solvent-extracted *M. pulmonis* were low (1:4 to 1:16) relative to *M. pneumoniae* (1:128 to 1:256) at similar concentrations of organisms (8); (ii) the lipid antigen of *M. pulmonis* was not specific but has been shown to react with antisera produced in rabbits against other *Mycoplasma* species, whereas the lipid component of *M. pneumoniae* was specific (7); (iii) *M. pulmonis* lipid preparations reacted only with rabbit antisera and not at all with sera from patients having had *M. pneumoniae* infection: (iv) rabbits showing preimmunization antibody titers to *M. pneumoniae* or *M. pulmonis* or from which *M. pulmonis* isolates had been obtained did not show antibody to the specific heat-stable antigenic components of 47 and 63. It is apparent then that the major antigenic component extracted from *M. pulmonis* by organic solvents is nonspecific in nature and is probably derived from the serum component of the medium (7).

Antigen lability studies with agar-gel double-diffusion tests. An unexpected result of the previous study was the finding that each of the rabbit strains of *M. pulmonis* could be distinguished by its own specific heat-stable, water-soluble antigen. Agar-gel double-diffusion tests were performed to determine the specificity of the boiled antigens of 47, 63, and 14267. Each of the three strains did possess at least one heat-stable diffusible antigen which formed precipitation lines only with homologous antiserum and which retained precipitating activity when diluted 1:4. To ascertain the nature of this component, periodate oxidation and pronase digestion were carried out on boiled and "raw" as well as "protein" antigens (trichloracetic acid precipitates of delipidized concentrates) for the rabbit strains. The heat-stable antigen was found to be stable to periodate, but it lost activity when subjected to pronase. "Raw" antigens treated in the same way were found to possess both periodate-stable, periodate-labile and pronase-labile, pronase-stable antigenic components. It must be noted that $NaIO_4$ solution alone did not make precipitation lines with serum on Immuno-Plates. Pronase alone did not make lines with antisera on Immuno-Plates and did not interfere with formation of precipitin lines. This conclusion was based on the fact that one precipitation line formed with pronase digested "raw" antigens, and this line remained stable on the plate for at least 1 week (Fig. 1 and 2). Since the precipitation lines formed by the pronase-stable components of each strain were found to fuse, it may be assumed that they were cross-related. These lines appeared at the same place in the agar as an antigen in "raw" concentrates, but may have been altered in some way or "uncomplexed," as judged by the spur formation at the point of intersection. "Raw" antigens diluted 1:2 with distilled water and used as controls in this procedure made only two or three precipitation lines with homologous antiserum. Two were specific for 63; one was specific for 47; and one line was shared by both strains and appeared in the same area on the plate as the periodate-labile, pronase-stable line. A subtype-specific, periodate-stable component appeared in about the same area as the specific antigens of whole concentrates. This antigen was obviously not identical, however, as indicated by the spur at the juncture, and may represent only one component of a complexity of precipitation lines in the region of the subtype-specific bands. When concentrates were crudely separated into "protein" and "polysaccharide" fractions by trichloroacetic acid, only the "protein" fraction retained any activity, and the precipitation lines formed by this fraction fused both with the lines formed by the specific boiled antigens and the subtype-specific lines of raw antigen. This "protein" fraction was also found resistant to periodate and labile to pronase treatment. When this fraction was boiled for 30 min, the 47 antigen was hydrolyzed into two precipitating components, whereas the 63 antigen retained only slight activity.

Antisera against boiled (B) antigens. Antisera prepared against boiled antigens of 63 and 47 were found to react with the specific heat-stable antigen of each organism, as well as with some cross-related antigens not detectible in the boiled immunizing concentrate itself. Antiserum against 63 B antigen made two precipitation lines with 63 B and one with 47 B. Antiserum against 47 B antigen made three lines with 47 B and two with 63 B. One of the lines with 63 B fused with one for 47. Antisera against "raw" antigens of 47 and 63 made only one diffuse line with homologous B antigen. However, when the antisera against

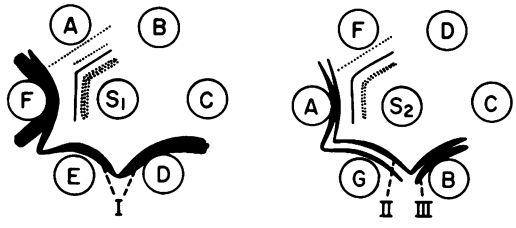

FIG. 1. *Diagrammatic representation of precipitin lines obtained when heat-treated antigens prepared from the three strains of Mycoplasma pulmonis were tested against immune sera. S_1, antisera to 47 strain; S_2, antisera to 63 strain; A, "raw" 63 antigen; B, boiled 63 antigen; C, boiled 14267 antigen; D, boiled 47 antigen; E, 47 "protein" antigen; F, "raw" 47 antigen; G, 63 "protein" antigen. Lines I, II, and III represent the subtype-specific, heat-stable antigens which were found to be resistant to >0.1 M $NaIO_4$ and to be inactivated by 100 μg of pronase.*

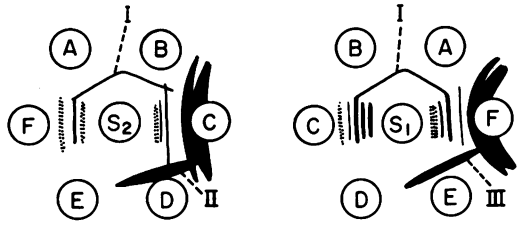

FIG. 2. *Diagrammatic representation of precipitin lines obtained when chemically treated antigens prepared from three strains of Mycoplasma pulmonis were tested against immune sera. S_1, antisera to 47 strain; S_2, antisera to 63 strain; A, 63 antigen pronase-treated ("raw" antigen diluted 10:1 with 1,000 μg/ml of pronase solution); B, 47 antigen pronase treated (as for 63); C, 63 "raw" antigen; D, 63 periodate-treated antigen ("raw" antigen diluted 1:2 with saturated $NaIO_4$ solution and incubated at room temperature for 4 hr—control "raw" antigens diluted with water only made only two to three lines with homologous antisera); E, 47 periodate-treated antigen (as for 63); F, 47 "raw" antigen. Line I represents a cross-related, periodate-labile, pronase-stable antigenic component. Lines II and III represent subtype-specific, periodate-resistant, pronase-sensitive antigens which appear to be related but not necessarily identical to the subtype-specific lines prior to treatment.*

boiled antigens were tested with "raw" antigens for the two strains, different precipitation patterns were observed. Four precipitation lines formed between anti-63 B and 63 "raw" antigen (two were very faint), and three formed with 47 "raw" antigen. Anti-47 B formed two faint but fusing lines with 47 and 63 "raw" antigens in addition to a line for each which was not identical with the other (Table 2). The results of these tests indicated that the subtype-specific antigens had been hydrolyzed by boiling into two or more antigenically active components, and that the antisera to boiled antigens lacked the specificity shown by antisera to "raw" antigens. It was further shown that antisera to boiled antigens were totally lacking in neutralizing ability, whereas controls of antisera to 63 or 47 "raw" antigens gave growth inhibition zones 4 to 5 mm wide for either strain.

TABLE 2. *Comparison of serological activity of antisera against boiled antigens and antisera against "raw" antigens of 47 and 63*[a]

Antigen	Antisera			
	Anti-63 "raw"	Anti-63 B	Anti-47 "raw"	Anti-47 B
63—"raw"..	5–6	4	5	3
63—B.....	1 wide band	2	0	2
47—"raw"..	3–5	3	6	3
47—B.....	0	1	1 wide band	3

[a] Results indicate precipitation lines on Immuno-Plates.

DISCUSSION

The results of this work may serve to point out two difficulties which arise when serotyping *M. pulmonis* strains by CF or immunodiffusion. (i) Cross-reactions with related organisms were demonstrated best with unheated "raw" antigens. (ii) Since the major CF antigen is subtype-specific, and the cross-related, water-soluble CF antigens react at lower dilutions, CF cross-typing is only possible by block titrations of antigens versus antisera.

Satisfactory boiled CF antigens have been demonstrated by Kenny and Grayston for *M. pneumoniae* (8), by Kenny for *M. fermentans* (7), and Lemcke for the bovine organism, *M. mycoides* (11). Other species of *Mycoplasma* from humans apparently lose CF specificity when heated to boiling (7, 11). In the above three examples, the heat-stable CF antigen was located in lipid extracts of concentrated suspensions, and did prove to be the specific and major CF component for each (7, 8, 14). Lemcke, in 1961 (9), reported that boiled and "fresh" antigens of *M. pulmonis* strains were equally satisfactory CF antigens against homologous antisera, but did not determine the nature of the heat-stable CF antigen. Our finding that the major heat-stable CF and precipitating antigen for *M. pulmonis* is not in lipid fractions but is water-soluble makes this species presently unique among the *Mycoplas-*

mataceae. The fact that this component is also subtype-specific makes it valuable as an antigenic marker and useful as a tool for subspecies identification of *M. pulmonis* strains.

The chemical lability studies included in this study would seem to give evidence for the existence of a cross-related precipitating polysaccharide antigen for the two rabbit strains, although the crude "polysaccharide" fraction, prepared as described, did not give precipitation lines. A water-soluble antigen (2) from *M. mycoides* has been identified as a galactan (12) and appears cross-related to an antigen of *M. Pneumoniae* on gel-diffusion (11).

The heat-labile, cross-related antigens are also of interest, because the antigen(s) responsible for the production of growth-inhibiting antibody apparently is found in this group. Since the heat treatment used in this study was probably excessive (boiling for 30 min), it would be interesting to discover the actual amount of heat by which neutralizing antigens are destroyed. If neutralizing antigens for *Mycoplasmataceae* are labile to even milder temperatures, then failures to produce neutralizing antisera may be attributed to denaturation of this antigen during growth or preparation of the immunizing antigen.

In conclusion, we would like to propose a serotyping scheme for strains of *M. pulmonis*. Members of the species *M. pulmonis* possess: (i) cross-related (species-specific), heat-labile antigens capable of eliciting neutralizing antibody response in immunized animals, (ii) subspecies-specific complement-fixing and precipitating antigens which are heat-stable, and probably proteins or protein complexes. This study reinforces the findings of Clyde that growth inhibition is the most specific means of identifying species of the family *Mycoplasmataceae* (1).

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LITERATURE CITED

1. CLYDE, W. A., JR. 1964. *Mycoplasma* species identification by specific antisera. J. Immunol. **92**:958–965.
2. DAFAALLA, E. N. 1957. A study of the antigenic structure of the contagious bovine pleuropneumonia organism. Bull. Epizoot. Diseases Africa **5**:135–145.
3. DEEB, B. J., AND G. E. KENNY. 1967. Characterization of *Mycoplasma pulmonis* variants isolated from rabbits. I. Identification and properties of isolates. J. Bacteriol. **93**:1416–1424.
4. FALLON, R. J., N. R. GRIST, D. R. INMAN, R. M. LEMCKE, G. NEGRONI, AND D. A. WOODS. 1965. Further studies of agents isolated from tissue cultures inoculated with human leukaemic bone-marrow. Brit. Med. J. **2**:388–391.
5. FOLCH, J., M. LEES, AND G. H. STANLEY. 1957. A simple method for the isolation and purification of total lipides from animal tissue. J. Biol. Chem. **226**:497–509.
6. JENSEN, K. E., L. B. SENTERFIT, R. M. CHANOCK, C. B. SMITH, AND R. H. PURCELL. 1965. An inactivated *Mycoplasma pneumoniae* vaccine. J. Am. Vet. Med. Assoc. **194**:248–253.
7. KENNY, G. E. 1966. Heat lability and organic solvent-solubility of *Mycoplasma* antigens. N.Y. Acad. Sci., *in press*.
8. KENNY, G. E., AND J. T. GRAYSTON. 1965. Eaton pleuropneumoniae-like organism (*Mycoplasma pneumoniae*) complement-fixing antigen: extraction with organic solvents. J. Immunol. **95**:19–25.
9. LEMCKE, R. M. 1961. Association of PPLO infection and antibody response in rats and mice. J. Hyg. **59**:401–412.
10. LEMCKE, R. M. 1964. The serological differentiation of *Mycoplasma* strains (pleuropneumoniae-like organisms) from various sources. J. Hyg. **62**:199–219.
11. LEMCKE, R. M. 1965. Related antigens in *Mycoplasma pneumoniae* and *Mycoplasma mycoides* var. *mycoides*. Australian J. Exptl. Biol. Med. Sci. **43**:761–770.
12. PLACKETT, P., AND S. H. BUTTERY. 1958. A galactan from *Mycoplasma mycoides*. Nature **182**: 1237.
13. STACEY, M., AND S. A. BARKER. 1960. Polysaccharides of microorganisms. Oxford Univ. Press, London.
14. YOSHIDA, T. 1961. Antigenicity of cell components of antigenic variants of contagious bovine pleuropneumonia organisms. Natl. Inst. Animal Health Quart. **1**:199–206.