J. Hyg., Camb. (1978), **80**, 357
with 3 plates
Printed in Great Britain

Serological and biochemical relationships between the alleged avirulent variant of *Corynebacterium kutscheri* and streptococci of group N

By R. G. HIRST* and R. J. OLDS
Department of Pathology, University of Cambridge, Cambridge, England

(*Received* 4 *October* 1977)

SUMMARY

Morphological, biochemical and serological observations suggested that no close relationship existed between *C. kutscheri* and its alleged avirulent variant isolated from mice at the Rockefeller University. However, both capillary and double diffusion precipitin reactions showed the alleged variant to be a streptococcus of group N, indistinguishable from that previously isolated from Rockefeller University mice.

INTRODUCTION

It has been suggested (Dubos, 1965*a*, *b*; Fauve, Pierce-Chase & Dubos, 1964; Pierce-Chase, Fauve & Dubos, 1964) that *Corynebacterium kutscheri*, a natural pathogen of rodent species, could exist in a latent state as an avirulent variant. These workers also claimed that this latent infection conferred on mice resistance to virulent *C. kutscheri* infection and that immunosuppression with hydrocortisone acetate induced a reactivated infection from which virulent *C. kutscheri* could be isolated.

In our laboratory, observations on these two organisms in resistant C57Bl/6 and susceptible Swiss Lynch mice have led to the conclusion that the alleged avirulent variant does not render susceptible mice resistant (Hirst & Olds 1978). Indeed, resistance of these mice to *C. kutscheri* infection has been shown to have a predominantly genetic basis (Hirst & Wallace, 1975, 1976).

An analysis by Bruce, Bismanis & Vickerstaff (1969) showed differences between *C. kutscheri* and its alleged avirulent variant in their cell wall content of arabinose and rhamnose, and in the buoyant density of their DNA. They observed that the alleged variant had many of the properties of a streptococcus. We present data which support these observations, and which show the alleged variant to be a streptococcus, probably of gastrointestinal origin.

* Present address: Department of Virology, Wellcome Research Laboratories, Beckenham, Kent, England.

MATERIALS AND METHODS

Bacterial strains

The *C. kutscheri* strains CMI, 9 and 23 were described previously (Hirst & Olds, 1978).

The streptococcal strains were obtained from the following sources:

GNS: from Dr C. Pierce-Chase, Rockefeller University, strain A; it had been isolated from the lung of a normal mouse and was considered to be an avirulent variant of *C. kutscheri* (Fauve *et al.* 1964; Pierce-Chase *et al.* 1964).

A739 and A740: from Dr R. Lancefield, Rockefeller University, as examples of group N streptococci – isolated from the gastrointestinal tract of mice at Rockefeller University (Schaedler, Dubos & Costello, 1965).

C 559: from Dr R. Lancefield, catalogued as NCTC6618; also known as the Orla-Jensen strain, a group N streptococcus.

Biochemical methods

The methods of Cowan & Steel (1965) were used for catalase, nitrate reduction and Christensen's urea tests. Other procedures were those of Cruickshank (1965). One to two per cent bovine serum was added where appropriate to improve the growth of streptococci.

Capillary precipitin tests

The procedure of Swift, Wilson & Lancefield (1943) was used.

Double diffusion precipitin tests

These tests were performed in agarose gel by the technique described by Ouchterlony (1967). Crude, neutralized, pH 2 extracts were placed in six peripheral wells and unabsorbed serum in a central well. The test was also run in the reverse orientation. The reagents reacted for 48 h at 4 °C in a moist environment. The slides were then washed in two changes of phosphate buffered saline over 48 h and dried overnight before staining with amido black.

Preparation of killed vaccines

Killed vaccines were prepared by the addition of formalin at a final concentration of 0·2 % to 100 ml of an overnight broth culture. After 2 days at 4 °C the organisms were sedimented, washed once, resuspended to the original volume in 0·1 % formol saline and stored at 4 °C.

Preparation of soluble precipitating antigens

Soluble antigens were prepared by Lancefield's method of extraction at pH 2 at 100 °C (Lancefield, 1933). The extracts were neutralized with 1 N-NaOH before removing the bacterial cells by centrifugation. This inhibited the formation of insoluble complexes between the antigen and other constituents of the extract which otherwise occurred at pH 2–3 (Ouchterlony, 1967).

Table 1. *Some biochemical characteristics of* C. kutscheri, *its alleged variant and group N streptococci*

	C. kutscheri CM1	Alleged variant GNS	Group N streptococci A739	A740	C559
Catalase	+	−	−	−	−
Nitrate	+	−	−	−	−
Urease	+	−	−	−	−
Sensitivity to 0·5 % deoxycholate	+	−	−	−	−
Trehalose	+	−	−	−	−

All produced acid from glucose, maltose, sucrose, laevulose and salicin, but not from lactose, galactose, arabinose, rhamnose, mannitol, dulcitol, adonitol or inositol.

Table 2. *Comparison of the capillary precipitin reactions of GNS with those of* C. kutscheri *and group N streptococci*

pH 2 extracts from organism	Antisera anti-CM23	anti-GNS	Anti-A739	anti-A740	anti-C559t‡	anti-C559gt§
CM1*	+	−	−	−	−	−
CM23*	+	−	−	−	−	−
GNS	−	+	+	+	−	+
A739†	−	+	+	+	−	+
A740†	−	+	+	+	−	+
C559†	−	−	−	−	+	+

* Typical *C. kutscheri*.
† Typical Group N streptococcus.
‡ Contains type-specific antibody.
§ Contains both group- and type-specific antibodies.

Antisera

Antisera were prepared in rabbits by methods previously described (Lancefield, 1933). Rabbits were used only if they had pre-immunization titres below 1/4 for agglutinating antibody. No precipitating antibody was found in any pre-immunization samples. Anti-C559gt (serum R1948) had group N and Orla-Jensen type antibodies, while anti-C559t (serum R708) had type antibodies only. Anti-A739 (serum R1851) and Anti-A740 (serum 1853) had type-specific antibodies only (Elliott, S. D., personal communication, 1974).

RESULTS

Cellular morphology

The gram-positive rods of *C. kutscheri* were usually slightly curved and tapered towards one end. Many metachromatic granules were demonstrated by Albert's method. Some rods occurred singly, while others were arranged in palisades, so-called Chinese letter forms, or in larger clumps. Their morphology was thus typical of that defined by Wilson & Miles (1974) for the genus Corynebacterium (Plate 1, fig. 1).

https://doi.org/10.1017/S0022172400024803 Published online by Cambridge University Press

On the other hand, its alleged variant GNS was considerably smaller, and it occurred in pairs (Plate 1, fig. 2 A) or sometimes chains (Plate 1, fig. 2 B). It was gram-positive, but it showed no metachromatic granules; morphologically it resembled a streptococcus.

Biochemical characteristics

A summary of some biochemical properties of *C. kutscheri*, its alleged variant and some streptococcal strains is shown in Table 1. While the alleged variant differed from *C. kutscheri* in trehalose, nitrate, urease and catalase reactions, and in sensitivity to 0·5% deoxycholate, its reactions in these tests are typical of those of the group N streptococci. For the other sugar fermentation tests described by Pierce-Chase *et al.* (1964) we found, as they did, that CM1 and GNS gave the same reaction; the group N streptococci behaved like GNS in these tests too.

Precipitin reactions

A positive reaction was observed when the extract from GNS was tested against anti-C559gt serum which had group N streptococcal specificity (Table 2). Moreover, the GNS extract also reacted with the type-specific antisera, anti-A739 and anti-A740. Similarly, extracts from the streptococci, A739 and A740, reacted equally well with anti-GNS. These results implied a close relation between GNS and the group N streptococci A739 and A740, isolated from the gastrointestinal tract of mice at the Rockefeller University. While extracts prepared from the *C. kutscheri* strains CM1 and CM23 gave good homologous reactions, they failed to react with either group or type streptococcal antisera. Trace reactions only were seen when streptococcal extracts were tested with anti-CM23, an anti-*kutscheri* serum.

Gel diffusion reactions

Acid extracts in peripheral wells were tested against unabsorbed antisera in central wells of micro-Ouchterlony plates; the results are presented in Plate 2. Anti-C559 serum in well A reacted with the homologous extract in well 1; there was a faint peripheral band of precipitation, and a denser inner one. Extracts from the group N streptococci, A739 in well 2 and A740 in well 4 and from GNS in well 3, all showed a single peripheral reaction of homology. We concluded that the peripheral reaction was group-specific, and the inner reaction was type-specific. This serum anti-C559gt gave no reactions with extracts prepared from *C. kutscheri* strains CM23 in well 5 and CM9 in well 6.

In well B, anti-C559t, serum known to be type-specific for Orla-Jensen strain C559, showed a homologous type reaction only.

The anti-*kutscheri* serum, anti-CM23 in well C, reacted strongly with the homologous extract in well 5 and with strain CM9 in well 6. Very faint heterologous reactions occurred with streptococcal extracts.

The group N streptococcus antiserum, anti-A739, in well D reacted with the group N extracts A739 and A740. Moreover, lines of identity were obtained with these two extracts and that from GNS. These reactions appeared to be type-specific, since no precipitation was observed using anti-A739 serum with group N

streptococcus extract C559. An identical precipitin pattern was obtained when anti-GNS in well E reacted with these extracts. Neither of the two anti-streptococcal sera, anti-C559gt nor anti-C559t, nor anti-GNS produced lines of precipitation with extracts from either of the two *C. kutscheri* strains.

The cross-reacting lines of precipitation between the streptococci and the *C. kutscheri* strains were of neither streptococcal group nor type specificity.

When these tests were run in the reverse orientation, similar results were obtained (Plate 3). Peripheral group and central type bands of precipitation were seen when strain C559 extract in well A was tested with anti-C559gt in well 1. A peripheral band, which denoted a type-specific reaction, was seen with anti-C559t in well 3. A faint band of precipitation with anti-CM23 in well 2 was unrelated to either of these group or type reactions.

Extract B prepared from the alleged variant GNS reacted with streptococcal type specific antisera A739 in well 4 and A 740 in well 6 and with the homologous anti-GNS in well 5, the lines of precipitation produced a typical reaction of identity which was type-specific. A group reaction was obtained with anti-C559gt in well 1. A thin band of precipitation was obtained with anti-CM23 in well 2, but it was unrelated to either group or type reactions of the streptococcal antisera.

Extract C prepared from *C. kutscheri* strain CM23 reacted only with the homologous antiserum in well 2, and produced a single dense central band.

DISCUSSION

The examination of virulent *C. kutscheri* and the avirulent organism received from the Rockefeller University showed no close serological or biochemical relationship to exist between them. Morphologically *C. kutscheri* was a typical corynebacterium, while the alleged variant resembled a streptococcus. Although common results were obtained in a series of sugar fermentation tests (Pierce-Chase *et al.* 1964), trehalose fermentation, catalase, urease, nitrate reduction and sensitivity to 0·5 % deoxycholate were all major differences. Moreover, the insensitivity of the avirulent organism to deoxycholate suggested that it might be of gut origin. Fauve *et al.* (1964) were unable to show any serological cross-reactivity between the two organisms by precipitin or double diffusion methods.

Apart from minor cross-reactions, we too found no evidence of antigenic relationship between the two bacteria. However, a close relationship between the alleged variant and group N streptotocci isolated from the gastrointestinal tract of mice at the Rockefeller University (Schaedler *et al.* 1965) was demonstrated by capillary precipitin reactions and by double diffusion tests in agarose.

Thus our strain GNS is quite typical of the parent culture previously described (Pierce-Chase *et al.* 1964); there was only one discrepancy between our observations on its cellular and colonial morphology and its biochemical and antigenic properties and those of Pierce-Chase *et al.* (1964). We found that it fermented trehalose, whereas they found that it did not; however, their method for determination of acid production was different from ours.

The rhamnose and arabinose content of streptococcal and corynebacterial cell

walls may be of taxonomic importance (Cummins & Harris, 1956); corynebacteria have less rhamnose than streptococci, but considerably more arabinose. Analysis for these sugars showed arabinose but not rhamnose in the cell walls of *C. kutscheri*, while the alleged avirulent variant contained only rhamnose (Bruce *et al.* 1969). There can be no doubt that strain GNS is a group N streptococcus: its cell wall sugars and the buoyant density of its DNA were similar to those of streptococci (Bruce *et al.* 1969); its antigens detected by the Ouchterlony method were identical with those of strains A739 and A740; so too were its morphology and biochemical reactions. The presence of group N streptococci in the normal flora of the gastrointestinal tract of mice at the Rockefeller University (Schaedler *et al.* 1965) suggests the probable source of the avirulent organism (Fauve *et al.* 1964).

Although the avirulent organism occurred naturally in mice at the Rockefeller University, it seemed to be no longer in these mice after they had been kept for 5 years at the Department of Pathology in Cambridge. Using experimental techniques similar to those described by Fauve *et al.* (1964) and Pierce-Chase *et al.* (1964) we readily isolated streptococci which were indigenous to our mice and almost certainly of gut origin. Although their extracts failed to react with Lancefield group A, B, C, D, G or N sera, they had many of the characteristics of group D streptococci (unpublished data).

It seems that the changed environment affected the gastrointestinal flora of our mice (Dubos *et al.* 1965); group D-like streptococci replaced the group N organisms which had been found in the mice at Rockefeller University.

In view of the lack of immunological cross-reaction between the two organisms, the group N streptococcus should not be expected to protect mice against challenge with *C. kutscheri* (Hirst & Olds, 1978). These results clearly support an earlier claim that in C57Bl/6 mice free from both *C. kutscheri* and the streptococcus, resistance to *C. kutscheri* is innate (Hirst & Wallace, 1975, 1976).

We are especially indebted to Dr Cynthia Pierce-Chase for the bacterial strains CM1 and GNS, and to Dr Rebecca Lancefield for the streptococcal strains A739 and A740

This work was undertaken while one of us (R. G. H.) was the holder of a Wellcome Trust Senior Veterinary Research Training Scholarship and was in receipt of a generous grant from the Marmaduke Shield Fund, Cambridge University.

REFERENCES

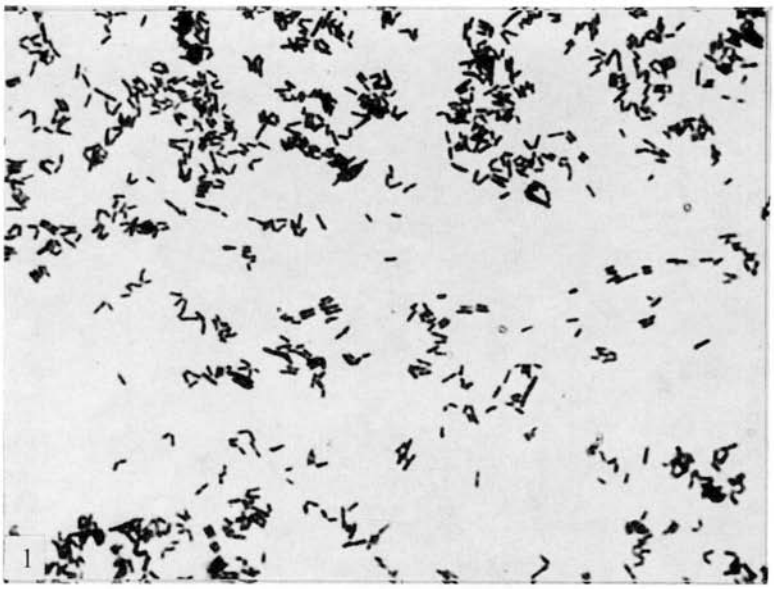
BRUCE, D. L., BISMANIS, J. E. & VICKERSTAFF, J. M. (1969). Comparative examinations of virulent *Corynebacterium kutscheri* and its presumed avirulent variant. *Canadian Journal of Microbiology* **15**, 817–18.

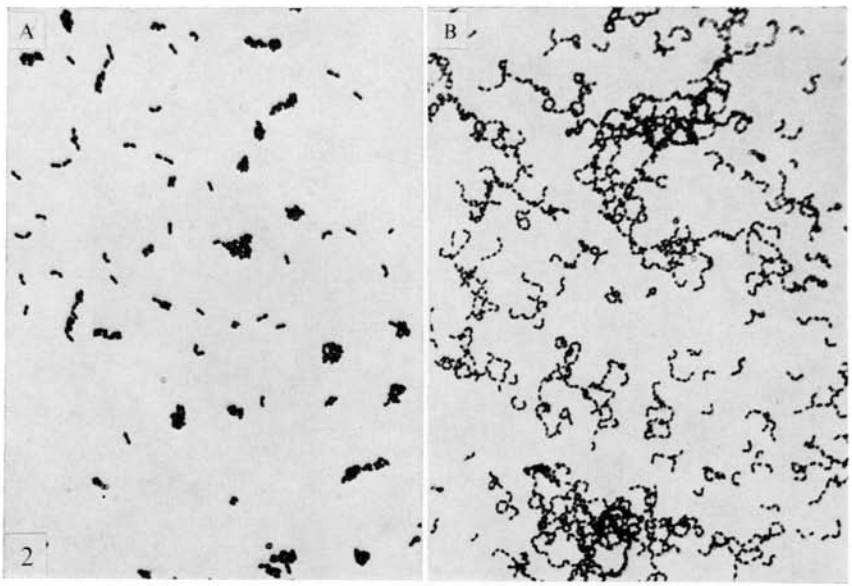
COWAN, S. T. & STEEL, J. K. (1965). *Manual for the Identification of Medical Bacteria*. Cambridge University Press.

CRUICKSHANK, R. (1965). *Medical Microbiology*. Edinburgh and London: E. & S. Livingstone.

CUMMINS, C. S. & HARRIS, H. (1956). The chemical composition of the cell wall in some gram-positive bacteria and its possible value as a taxonomic character. *Journal of General Microbiology* **14**, 583–600.

DUBOS, R. J. (1965*a*) *The Evolution of Microbial Disease in Bacterial and Mycotic Infections of Men* (ed. R. J. Dubos and J. G. Hirsch). London: Pitman Medical Publishing Co. Ltd.

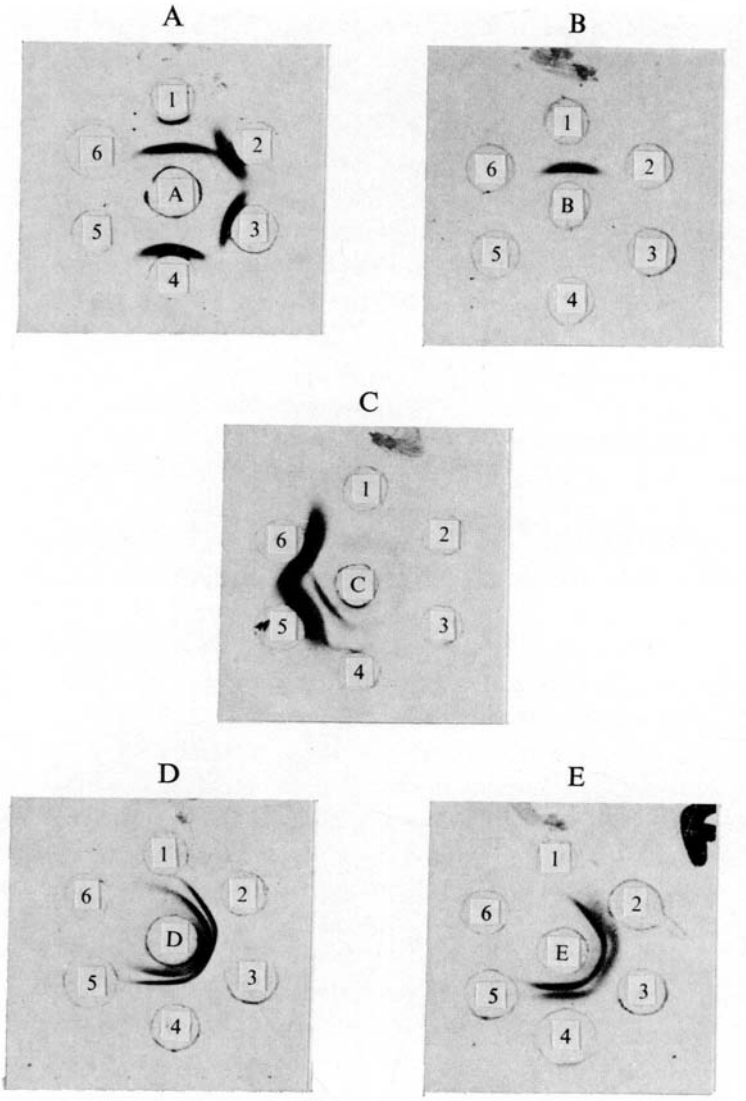




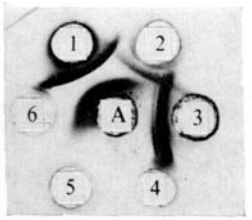
R. G. HIRST AND R. J. OLDS

(*Facing p.* 362)

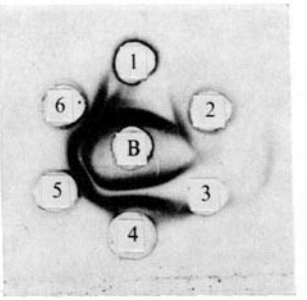
https://doi.org/10.1017/S0022172400024803 Published online by Cambridge University Press



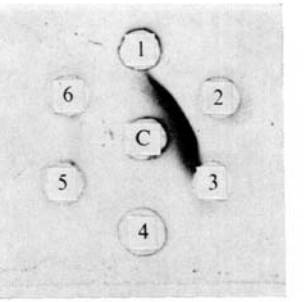
https://doi.org/10.1017/S0022172400024803 Published online by Cambridge University Press



Extract of group N streptococcus C559



Extract of the alleged avirulent variant, GNS



Extract of *Corynebacterium kutscheri*

https://doi.org/10.1017/S0022172400024803 Published online by Cambridge University Press

Dubos, R. J. (1965*b*) *Man Adapting*. New Haven, Conn.: Yale University Press.

Dubos, R. J., Schaedler, R. W., Costello, R. & Hoet, P. (1965). Indigenous, normal and autochthonous flora of the gastrointestinal tract. *Journal of Experimental Medicine* **122**, 67–76.

Fauve, R. M., Pierce-Chase, C. H. & Dubos, R. J. (1964). Corynebacterial pseudo-tuberculosis in mice. II. Activation of natural and experimental latent infections. *Journal of Experimental Medicine* **120**, 283–304.

Hirst, R. G. & Olds, R. J. (1978). *Corynebacterium kutscheri* and its alleged avirulent variant in mice. *Journal of Hygiene* **80**, 349–56.

Hirst, R. G. & Wallace, M. E. (1975). Resistance to a natural bacterial pathogen. *Mouse News Letter* **53**, 20–21.

Hirst, R. G. & Wallace, M. E. (1976). Inherited resistance to *Corynebacterium kutscheri* in mice. *Infection and Immunity* **14**, 475–82.

Lancefield, R. C. (1933). A serological differentiation of human and other groups of hemolytic streptococci. *Journal of Experimental Medicine* **57**, 571–95.

Ouchterlony, O. (1967). *Handbook of Experimental Immunology* (ed. D. M. Weir). Philadelphia: F. S. Davis & Co.

Pierce-Chase, C. H., Fauve, R. M. & Dubos, R. (1964). Corynebacterial pseudo-tuberculosis in mice. I. Comparative susceptibility of mouse strains to experimental infection with *Corynebacterium kutscheri*. *Journal of Experimental Medicine* **120**, 267–81.

Schaedler, R. W., Dubos, R. & Costello, R. (1965). The development of the bacterial flora in the gastrointestinal tract of mice. *Journal of Experimental Medicine* **122**, 59–66.

Swift, H. F., Wilson, A. T. & Lancefield, R. C. (1943). Typing group A hemolytic streptococci by M precipitin reactions in capillary pipettes. *Journal of Experimental Medicine* **78**, 127–33.

Wilson, G. S. & Miles, A. A. (1974). *Topley and Wilson's Principles of Bacteriology and Immunity*. London: Edward Arnold Ltd.

EXPLANATION OF PLATES

Plate 1

Fig. 1. *Corynebacterium kutscheri* showing typical coryneform morphology.

Fig. 2. The alleged atypical variant of *Corynebacterium kutscheri*. A, in pairs; B, showing typical streptococcal morphology.

Plate 2

Double diffusion reactions in agarose of unabsorbed antisera in the central wells: A = anti-C559gt, B = anti-C559t, C = anti-CM23, D = anti-A739, E = anti-GNS. The peripheral wells contain acid extracts of organisms as follows; 1, C559; 2, A739; 3, GNS; 4, A740; 5. CM23; 6, CM9.

Plate 3

Double diffusion reactions in agarose. Central wells contain pH 2 extracts of organisms: A = group N streptococcus C559; B = the alleged avirulent variant, GNS; C = *C. kutscheri*. The peripheral wells contain unabsorbed antisera: 1, anti-C559gt; 2, anti-CM23; 3, anti-C559t; 4, anti-A739; 5, anti-GNS; 6, anti-A740.