SHORT ARTICLE

Serotyping of campylobacters by co-agglutination on the basis of heat-stable antigens

C. R. FRICKER, J. URADZINSKI, M. M. ALEMOHAMMAD, R. W. A. PARK, C. WHELAN* and R. W. A. GIRDWOOD*

*Department of Microbiology, University of Reading, London Road, Reading RG1 5AQ, and *Department of Bacteriology, Stobhill General Hospital, Glasgow G21 3UW*

Summary. A new and simple method of serotyping campylobacters has been developed which utilises co-agglutination to detect the presence of heat-stable antigens. Campylobacters are heated at 75°C for 30 min to destroy antigenic protein and allowed to react on a glass slide with staphylococci coated with antibody. Of 74 isolates, 67 gave the same result by co-agglutination and the previously described passive haemagglutination method. The co-agglutination technique may be used as a rapid screening test before serotyping by passive haemagglutination.

Introduction

Campylobacter jejuni, and to a lesser extent *C. coli*, are major causes of morbidity in man (Skirrow, 1982), being the most frequently reported bacterial cause of diarrhoea in Britain and increasingly being associated with a wide range of other infections (Mandal *et al.*, 1984). Campylobacters are relatively inactive in the traditional physiological tests used in microbiology and so there is a need to develop effective serotyping methods for epidemiological studies. Several schemes have been reported in the literature (Penner and Hennessy, 1980; Lauwers *et al.*, 1981; Itoh *et al.*, 1982; Lior *et al.*, 1982; Rogol *et al.*, 1982).

The Lior scheme is based upon heat-labile antigens and utilises slide agglutination for detection of antigen-antibody reactions. The Penner scheme is based on saline extractable, heat-stable antigens (thought to be lipopolysaccharide), the antigen-antibody reactions being detected by passive haemagglutination. Combined use of both systems has been useful in epidemiological studies (Patton *et al.*, 1983).

The aim of this study was to develop a simple and rapid screening test for serotyping clinical isolates of campylobacters on the basis of heat stable (Penner) antigens. Slide agglutination tests have been shown to lack specificity with regard to the heat stable antigens of campylobacters (Penner and Hennessy, 1980); therefore we attempted to develop a technique for such antigens based on co-agglutination, a technique which has previously been suggested for serotyping campylobacters (Kosunen *et al.* 1980).

Materials and methods

Campylobacter strains

Twenty-five *Campylobacter* reference strains corresponding to Penner serotypes (Pen) 1–4, 6–11, 13, 15, 16, 18–21, 24, 27–29, 31, 37, 44 and 55 were used. Another 74 strains belonging to the *C. jejuni-coli* group which had

been isolated from human faeces and serotyped at the Department of Bacteriology, Stobhill General Hospital, Glasgow as belonging to Penner serotypes 1, 2, 4, 9, 11, 15, 18, 20, 24 or 27 were used. Cultures were stored at –70°C, as suspensions from blood-agar plates in nutrient broth no. 2 (Oxoid CM67) containing glycerol 10% v/v and FBP supplement (George *et al.*, 1978).

Production of antisera

Organisms were cultivated on freshly prepared blood-agar plates (Fricker, 1985) held at 42°C for 48 h in an atmosphere of reduced oxygen (O₂ 5%, CO₂ 10%, N₂ 85%). Growth was harvested into sterile saline (0.85%), washed twice and resuspended in sterile saline to an absorbance of approximately 0.4 at 625 nm, measured on a Perkin Elmer 550S spectrophotometer. Rabbits were given intravenous inocula five times over a 2-week period of 1.0, 2.0, 4.0, 4.0 and 4.0 ml, respectively, the last injection containing double the concentration of cells. Blood was collected by cardiac puncture 7 days after the last inoculation and allowed to clot. Serum was collected and stored at –70°C. The passive haemagglutination titre of each serum was determined by the method of Penner and Hennessy (1980).

Preparation of reagent staphylococci

The procedure used was essentially that described by Kronvall (1973). A Cowan I strain of *Staphylococcus aureus* was inoculated into 1.2 L of Tryptone Soya Broth (Oxoid CM129) in a 2-L conical flask and incubated at 37°C for 24 h in a shaking incubator. Cells were harvested by centrifugation at 10 000 g for 10 min and washed twice with phosphate buffered saline (PBS), pH 7.2. The cell pellet was then resuspended to 10% w/v in PBS containing formaldehyde 0.5% and incubated at room temperature for 3 h with constant stirring. The cells were washed three times with PBS, resuspended to 10% w/v and heated at 80°C for 1 h. After washing twice in PBS they were resuspended to 10% w/v in PBS containing sodium azide 0.1%.

Preparation of antibody-coated staphylococci

Doubling dilutions (2–16) of each of the ten antisera raised against Penner reference strains of *Campylobacter* (types 1, 2, 4, 9, 11, 15, 18, 20, 24, 27) were prepared in PBS. Undiluted serum (0.1 ml) and 0.1 ml of each dilution were added to separate 1.0 ml volumes of reagent staphylococci, mixed, incubated at room temperature for 10 min and washed in PBS. Cells were then resuspended in PBS to 10% w/v and held at 4°C until required. All antibody-coated staphylococci were used within 7 days of preparation.

Preparation of soluble antigens

After incubation for 48 h at 42°C, blood-agar cultures of the 25 Penner reference strains were harvested into sterile saline to an optical density equivalent to Brown's tube 10 and heated at 100°C for 1 h. The supernatant fluids were collected after centrifugation at 10 000 *g* for 5 min and used as antigens.

Preparation of cellular antigens

Cultures of the 25 Penner reference strains were harvested and suspended as described above. Each suspension (1.0 ml) was then heated at 65°C, 75°C, 85°C and 100°C for 30 min. The cells were then sedimented by centrifugation, washed in PBS and resuspended in 0.3 ml of PBS for use as antigens.

Titration of antisera against reference antigen preparations

One loopful (*c.* 3 μ l) of antigen preparation was mixed with an equal volume of antibody-coated staphylococci on a glass slide. The suspension was mixed by gently rocking the slide for 1 min, after which it was viewed for agglutination. The five staphylococcal reagents prepared for the ten antisera available were tested against each of their homologous antigen preparations. The highest dilution of each serum which showed a strong reaction with each type of antigen preparation was then tested against the remaining 24 antigen preparations which had been produced in the same way.

Serotyping of 74 clinical Campylobacter isolates

Recent clinical *Campylobacter* isolates were typed by the haemagglutination method of Penner and Hennessey (1980). The haemagglutination serotyping was performed in Glasgow and the results were not divulged to the Reading workers until they had completed their studies of co-agglutination. Organisms were grown on blood-agar plates for 48–72 h, harvested into sterile PBS to an optical density equivalent to Brown's tube 10 and heated at 75°C for 30 min. The cells were then washed, resuspended in PBS and tested against antibody-coated staphylococci, as described above. All strains, including those which gave different results with the two serotyping procedures were typed in duplicate by different workers.

Results

Homologous soluble antigens reacted with staphylococci coated with undiluted antiserum, but not with staphylococci coated with diluted serum. The reactions were not serotype specific, and many cross reactions with heterologous antigen preparations occurred. This procedure was therefore considered to be of little practical use.

Experiments to determine whether heated cells could be used as antigens yielded some interesting results. Our hypothesis was that by heating the cells antigenic protein would be destroyed whilst the heat-stable antigens would remain intact. Cells treated at 65°C reacted strongly with each of the five dilutions of serum but also with many heterologous antisera, and this suggested that some antigenic protein had not been destroyed. Cells heated at 85°C or 100°C reacted less strongly with higher dilutions of homologous antisera, gave some reactions with heterologous sera, and tended to autoagglutinate, making detection of co-agglutination difficult. Cells treated at 75°C, however, reacted with their homologous antisera (although not always with the 1 in 8 and 1 in 16 dilutions) and showed only weak cross reactivity, easily distinguishable from the strong reactions obtained with homologous sera. One reference strain, Pen 15, reacted strongly with eight of the ten antibody preparations. The dilutions of serum used for typing clinical isolates were chosen by selecting the highest dilution of serum that gave a strong reaction with the homologous heated-cell preparation. The dilution of each serum used, and the passive haemagglutination titre of each serum are shown in table I.

When cells from cultures younger than 48 h were heated at 75°C, a gelatinous mass was sometimes formed, and, therefore, 48–72 h cultures were used for typing clinical isolates. Of the 74 such isolates tested, 67 gave a result which agreed with the haemagglutination (Penner) result, two gave a different serotype with the two systems and five did not react in co-agglutination tests (table II).

Table I. Dilutions of antiserum used in serotyping clinical isolates and passive haemagglutination titre of each serum

Reference strain used to raise antiserum	Dilution used for co-agglutination	Passive haemagglutination titre (undiluted serum)
Pen 1	4	1280
Pen 2	4	320
Pen 4	4	1280
Pen 9	8	10 240
Pen 11	4	1280
Pen 15	16	5120
Pen 18	16	2560
Pen 20	4	2560
Pen 24	1	5120
Pen 27	16	2560

Pen = Penner serotype.

Table II. Comparison of passive haemagglutination and co-agglutination in serotyping clinical campylobacter isolates

Serotype identified		Number of isolates giving this result
by passive heamagglutination	by co-agglu- tination	
<i>Concordant results</i>		
Pen 1	Pen 1	15
Pen 2	Pen 2	18
Pen 4	Pen 4	9
Pen 9	Pen 9	4
Pen 11	Pen 11	4
Pen 15	Pen 15	3
Pen 18	Pen 18	3
Pen 20	Pen 20	3
Pen 24	Pen 24	3
Pen 27	Pen 27	5
<i>Discordant results</i>		
Pen 1	Pen 2	1
Pen 2	Pen 1	1
Pen 2	not typable	2
Pen 4	not typable	2
Pen 24	not typable	1

Pen = Penner serotype.

The same results were obtained by different workers on two separate occasions.

No loss of reactivity was seen in antibody-coated staphylococci which had been stored at 4°C for 7 days. Staphylococci stored for longer periods were not tested.

Discussion

Co-agglutination has been successfully applied to the serotyping of pneumococci (Kronvall, 1973), meningococci (Danielsson and Olcen, 1979) and gonococci (Danielsson and Kronvall, 1974) and it has now been shown to be appropriate to campylobacters.

Use of campylobacters heated at 75°C for 30 min as antigen gives results in close agreement with those from the passive haemagglutination procedure (Penner and Hennessy, 1980). The technique is simple and rapid, giving few cross reactions. It is not known why the reference strain of Pen 15, but not the three recent isolates of Pen 15, reacted with heterologous antisera in co-

agglutination but not in haemagglutination, or why serum raised against Pen 15 did not cross react with other reference strains, although (in the latter case) quantitative rather than qualitative differences in antigen may be involved (Morgan, 1959).

Frequent cross reactions when saline extracts were used as antigens were unexpected, but perhaps each extract contains at least two antigenic components. One might adsorb to sheep erythrocytes and impart serospecificity in the passive haemagglutination system while another, apparently 'common' component may not adsorb to erythrocytes (or may adsorb, but not react with antisera) but may react in co-agglutination tests. Abbott *et al.* (1980) had to cross absorb each of their hyperimmune sera raised against heated campylobacters because of the many cross reactions which occurred, indicating "common" antigens. Perhaps some of these "common" antigens are released into solution when cell suspensions are heated in saline and so cause cross reactions in co-agglutination tests.

Campylobacters heated at 85°C or 100°C lacked immunological specificity, as found by Penner and Hennessy (1980), and tended to autoagglutinate, as found by Berg *et al.* (1971). Cross reactivity was also seen with cells heated at 65°C, presumably because of some remaining intact protein, but cells heated at 75°C showed no cross reactivity (with the exception of Pen 15) and did not autoagglutinate.

Current techniques for serotyping campylobacters on the basis of their heat-stable antigens are labour intensive and time consuming. The Penner scheme recognises more than 50 serotypes and some laboratories prepare 'pools' of antisera to screen each isolate before titration of individual antisera, which saves reagents but increases the time needed to obtain a definitive result. Co-agglutination reduces the time taken to serotype clinical isolates and removes the requirement for a regular supply of sheep erythrocytes. We are now using the method as a screening test and are extending the range of serotypes which can be detected. Co-agglutination requires no special reagents or equipment and should facilitate more widespread serotyping of campylobacters.

This work was supported by the Ministry of Agriculture, Fisheries and Food (Reading) and the Scottish Home and Health Department (Glasgow). J. Uradzinski was supported by a British Council Fellowship. M. M. Alemohammad was supported by the Iranian Government.

REFERENCES

- Abbott J D, Dale B, Eldridge J, Jones D M, Sutcliffe E M 1980 Serotyping of *Campylobacter jejuni/coli*. *Journal of Clinical Pathology* **33**:762-766.
- Berg R L, Jutila J W, Firehammer B D 1971 A revised classification of *Vibrio fetus*. *American Journal of Veterinary Research* **32**:11-22.
- Danielsson D, Olcen P 1979 Rapid serotyping of groups A, B and C meningococci by rocket-line immunoelectrophoresis and co-agglutination. *Journal of Clinical Pathology* **32**:136-142.
- Danielsson D, Kronvall G 1974 Slide agglutination method for the serological identification of *Neisseria gonorrhoeae* with anti-gonococcal antibodies adsorbed to protein A-containing staphylococci. *Applied Microbiology* **27**:368-374.
- Fricker C R 1985 A note on the effect of different storage procedures on the ability of Preston medium to recover campylobacters. *Journal of Applied Bacteriology* **58**:57-62.

- George H A, Hoffman P S, Smibert R M, Krieg N R 1978 Improved media for growth and aerotolerance of *Campylobacter fetus*. *Journal of Clinical Microbiology* **8**:36–41.
- Itoh T, Saito K, Yanagawa Y, Sakai S, Ohashi M 1982 Serological typing of thermophilic campylobacter isolated in Tokyo. In: Newell D G (ed) *Campylobacter: Epidemiology, pathogenesis and biochemistry*. MTP Press, Lancaster, pp 106–110.
- Kosunen T U, Danielsson D, Kjellander J 1980 Serology of *Campylobacter fetus* ss *jejuni* ('related campylobacters'). *Acta Pathologica, Microbiologica et Immunologica Scandinavica B*. **88**:207–218.
- Kronvall G 1973 A rapid slide-agglutination method for typing pneumococci by means of specific antibody adsorbed to protein A-containing staphylococci. *Journal of Medical Microbiology* **6**:187–190.
- Lauwers S, Vlaes L, Butzler J P 1981 *Campylobacter* serotyping and epidemiology. *Lancet* **1**:158–159.
- Lior H, Woodward D L, Edgar J A, Laroche L J, Gill P 1982 Serotyping of *Campylobacter jejuni* by slide agglutination based on heat-labile antigenic factors. *Journal of Clinical Microbiology* **15**:761–768.
- Mandal B K, De Mol P, Butzler J P 1984 Clinical aspects of *Campylobacter* infections in humans. In: Butzler J P (ed) *Campylobacter infection in man and animals*, CRC Press, Florida. pp 21–31.
- Morgan W J B 1959 Studies on the antigenic structure of *Vibrio fetus*. *Journal of Comparative Pathology and Therapeutics* **69**:125–140.
- Patton C M, Barrett T J, Morris G K 1983 Serotyping *Campylobacter jejuni/coli* by two systems: the CRC experience. In: Pearson AD *et al.* (eds) *Campylobacter II*, Public Health Laboratory Service, London, pp 96–97.
- Penner J L, Hennessy J N 1980 Passive hemagglutination technique for serotyping *Campylobacter fetus* subsp *jejuni* on the basis of soluble heat-stable antigens. *Journal of Clinical Microbiology* **12**:732–737.
- Rogol M, Sechter I, Braunstein I, Gerichter C B 1982 Provisional antigenic scheme for *Campylobacter jejuni*. In: Newell DG (ed) *Campylobacter: Epidemiology, pathogenesis and biochemistry*, MTP Press, Lancaster. pp 98–103.
- Skirrow M B 1982 *Campylobacter* enteritis—the first five years. *Journal of Hygiene*, **89**:175–184.