

Typing of *Clostridium difficile* by Western Immunoblotting with 10 Different Antisera

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Western blotting (immunoblotting) with antisera against each of 10 reference serogroups was evaluated as a means of typing *Clostridium difficile*. A total of 164 clinical isolates of *C. difficile* were tested. Variations in band profiles in each serogroup were used to type isolates into subserogroups. This technique was useful for an epidemiological investigation.

Outbreaks of *Clostridium difficile*-associated diarrhea in hospitalized patients are well documented (3, 5, 7, 8, 10). The availability of a simple, rapid, reproducible, and highly discriminatory typing scheme for *C. difficile* would greatly aid studies of both the epidemiology and pathogenicity of *C. difficile*. Various typing methods have been reported to date (3, 5, 7-10, 13). Of these, Western blotting (immunoblotting) results have been reported to be relatively simple to interpret and the test is sufficiently sensitive for typing of epidemiologically related *C. difficile* isolates (9, 10). The studies used Western blotting as the typing method and antiserum prepared against one strain of *C. difficile* (10) or antisera prepared against two clinical isolates (9). However, typing into serogroups that are defined by a slide agglutination test (5, 6) is likely more beneficial in terms of the epidemiology and pathogenicity of *C. difficile*. Therefore, in the present study, we prepared antisera against the 10 reference serogroups of *C. difficile*. These antisera were used to type clinical isolates by the Western blotting technique.

Ten reference strains of *C. difficile* representing the following 10 different serogroups (5, 6) were obtained from the American Type Culture Collection: A, ATCC 43594; B, ATCC 43593; C, ATCC 43596; D, ATCC 43597; F, ATCC 43598; G, ATCC 43599; H, ATCC 43600; I, ATCC 43601; K, ATCC 43602; X, ATCC 43603. A total of 164 *C. difficile* strains isolated from clinical specimens were tested. Of the 164 strains, 114 were isolated from epidemiologically unrelated patients; the strains were received by the Anaerobic Bacteria Branch, Centers for Disease Control, between 1984 and 1986 and were from various sources. The remaining 50 strains were isolated from 36 patients associated with a presumed outbreak of *C. difficile*-associated colitis or diarrhea at a hospital in New York in 1989 and 1990. All 50 isolates were recovered from fecal specimens from symptomatic adult patients. *C. difficile* was isolated and identified by standard procedures (1, 2). Antisera were prepared by immunizing New Zealand rabbits by the method described previously (5) by using each of the reference strains of the 10 different serogroups. Protein antigen was prepared by EDTA extraction of *C. difficile* (13). Electrophoresis of EDTA

extracts was performed on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system consisting of a 10% separating gel and a 5% stacking gel. Following SDS-PAGE, the separated proteins were transferred to a nitrocellulose membrane as described previously (12). The membranes were incubated with goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase, and color development became visible by soaking the membrane with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium.

Western blotting patterns for the 10 reference strains with homologous antisera showed different profiles, with distinctive bands for each serogroup located in 30- to 60-kDa range (data not shown). Because it was not practical to type the *C. difficile* isolates by the Western blot technique with 10 separate antisera, isolates were first screened by using two antiserum pools (each pool containing five antisera) for immunoblotting; antiserum pool 1 consisted of serogroups A, B, F, G, and X, and antiserum pool 2 comprised serogroups C, D, H, I, and K. To prove that the two antiserum pools were effective for immunoblotting, the 10 reference strains were tested. Specific and strong immunoblot patterns were observed for each serogroup when the antiserum pools were reacted with proteins of the corresponding homologous reference strains (Fig. 1a and d). The patterns observed for each reference strain were very similar to those seen when a specific antiserum alone was reacted with its homologous reference strain. When the antiserum pools were immunoblotted against heterologous reference strains, minor bands were demonstrated, but strain specificity was absent (Fig. 1b and c). When we used this procedure and then confirmatory immunoblotting with a specified antiserum which appeared to react specifically with the isolate, we were able to serotype 68 (59.6%) of the 114 unrelated clinical isolates into eight serogroups; 46 (40.4%) of the isolates were classified as nontypeable; i.e., immunoblotting did not show specific bands in the 30- to 60-kDa region with any of the 10 antisera. The most common serogroup isolated was serogroup H (22 [19.2%] of 114 isolates). No representative of either serogroup B or serogroup F was found. A total of 14 isolates were recovered from patients who were diagnosed as having pseudomembranous colitis by endoscopic examination. Two of these isolates were typed as serogroup A, one isolate was typed as serogroup C, two isolates were typed as serogroup G, three isolates were typed as serogroup K, and six isolates

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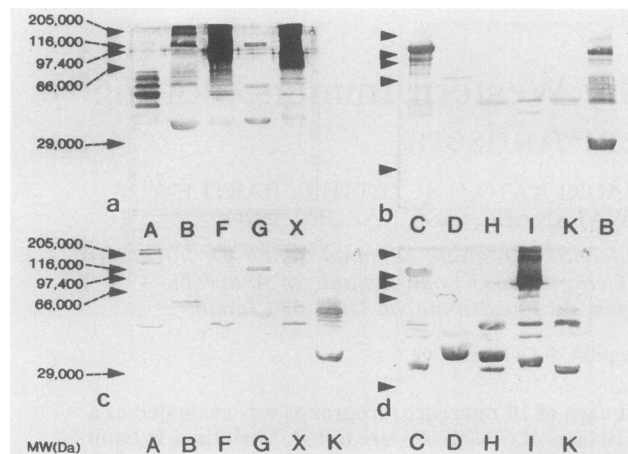


FIG. 1. Western blotting patterns of 10 reference strains of *C. difficile*. (a and b) Immunoblotting patterns with antiserum pool 1, consisting of antisera against the reference strains of serogroup A, B, F, G, and X. (c and d) Immunoblotting patterns with antiserum pool 2, consisting of antisera against the reference strains of serogroup C, D, H, I, and K. The letter below each lane refers to the serogroup of the strain used for Western blotting. By using only the combination of the reference strains and their homologous antisera, strong immunoblotting patterns were seen in the region of 30 to 60 kDa (a and d); serogroup B (lane B, panel b) and serogroup K (lane K, panel c) were homologous controls. MW, molecular weight.

were nontypeable. When we used homologous antiserum, there were variations in immunoblot patterns among the strains belonging to the same serogroup, with bands located beyond the region of 60 kDa. These variations showed reproducibility after several subcultures. Therefore, these differences were used to further type the isolates into subserogroups within a serogroup. Representative immunoblot patterns of serogroup K isolates with homologous serogroup K antiserum are shown in Fig. 2; depending on the locations of the bands of greater than 60 kDa, the isolates tested displayed eight different profiles. Of the 114 unrelated clinical isolates, 68 were further typed into 22 subserogroups.

Fifty *C. difficile* isolates were obtained from 36 patients involved in what was believed to be a hospital outbreak. A total of 34 of these isolates were typed as serogroup G and of these, 33 (66%) were typed as subserogroup G-1; these 33 strains were isolated from 28 (78%) of the 36 patients. *C. difficile* was repeatedly isolated from more than two different

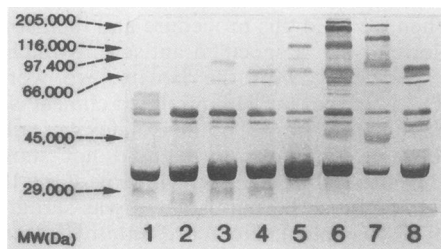


FIG. 2. Western blotting patterns of clinical isolates which were typed as belonging to serogroup K by using anti-serogroup K antiserum (1:800). Variations in bands observed in the region with molecular sizes of greater than 60 kDa was used to type serogroup K isolates into eight subserogroups. Lane 1, reference strain of serogroup K; lanes 2 to 8, clinical isolates. MW, molecular weight.

fecal specimens from nine patients. In three of these nine patients, the serogroups of the isolates changed from one to another during their hospitalization.

In the present study, the application of immunoblotting with rabbit antisera prepared against each of the 10 reference serogroup strains consistently demonstrated the presence of easily identifiable serogroup-specific protein bands in the region of 30 to 60 kDa. The method was able to differentiate between the 10 serogroups of *C. difficile* and to do so reproducibly. Common bands among *C. difficile* strains were not demonstrated (4). In our study, the most commonly recovered strains from epidemiologically unrelated isolates belonged to serogroups A, G, H, and K. These results confirm the previous observations of Toma et al. (11). In addition, the present study showed additional minor bands in the region beyond 60 kDa. These bands were reproducible and useful in typing the different isolates into subserogroups.

The results of our laboratory investigations supported the fact that there was an outbreak of *C. difficile* infection among the hospitalized patients we examined; one of the serogroup G subgroups, subserogroup G-1, was recovered from 78% of patients; on the other hand, strains of this subserogroup were isolated from only 7.0% of the patients not involved in the outbreak. We concluded that this subserogroup strain was the causative agent of the outbreak.

The 10 serogroups of *C. difficile* were characterized (5, 6), and the reference strains are available from the American Type Culture Collection. Once antisera against the reference serogroup strains have been produced which cannot easily be performed in clinical laboratories, Western blotting seemed to be a potent method of typing *C. difficile* and could offer a means of exchanging typing results among laboratories. One limitation of the Western blotting technique for typing is the number of antisera needed for serogrouping. In the present study, some isolates could not be typed with the available antisera. Establishment of additional serogroups of *C. difficile* is required to make new antisera and to make the Western blotting technique more useful for typing.

In conclusion, we demonstrated that the Western blotting technique is a sensitive and viable method for typing *C. difficile*, although we found appreciable numbers of strains that could not be typed. The method was able to differentiate different *C. difficile* isolates and allow the determination of the causative agent of an outbreak. The relationship and correlation of serogroups or subserogroups to toxigenicity, pathogenicity, and clinical symptomatology of *C. difficile* infections warrant additional studies.

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