Clinical and Laboratory Diagnostic Characteristics and Cytotoxigenic Potential of Hafnia alvei and Hafnia paralvei Strains

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A collection of 68 Hafnia strains previously identified to the species level by 16S rRNA gene sequencing were investigated for simple phenotypic properties that could aid in their recognition in the clinical laboratory. Four tests, including malonate utilization, fermentation of salicin and D-arabinose, and expression of β-glucosidase activity, correctly assigned each strain to either Hafnia alvei or H. paralvei. Antibiotic susceptibility profiles were generated for 35 H. alvei and H. paralvei isolates using Etest strips for 24 antibiotics. All strains were susceptible to aminoglycosides, quinolones, carbapenems, and monobactams. Most of the Hafnia isolates had a colistin MIC of ≥2 μg/ml. Sequencing of an internal ampC gene fragment allowed genotypic differentiation of the two Hafnia species. Approximately 70% of the hafniae tested additionally produced a cytolytic toxin active on Vero cells which may play a role in gastroenteritis.

The genus Hafnia is one of more than 40 genera that currently make up the family Enterobacteriaceae (14). While it is often considered to be a miscellaneous member of this group, an increasing awareness of the possible role hafniae may play in causing human and animal diseases has emerged over the past few years (15). Recent investigations have focused on associations between the genus Hafnia and emerging antimicrobial resistance patterns (22, 23, 24) and infections associated with unusual disease presentations such as hemolytic-uremic syndrome (HUS) (7), graft-versus-host disease (23), and stem cell or tissue transplantation studies (1, 5). Some associations between the genus Hafnia and emerging antimicrobial resistance patterns (22, 23, 24) and infections associated with unusual disease presentations such as hemolytic-uremic syndrome (HUS) (7), graft-versus-host disease (23), and stem cell or tissue transplantation studies (1, 5). Some reports even suggest that hafniae are candidate enteric pathogens, although very limited data and evidence is currently available in this area (8).

Since the pioneering taxonomic studies of Steigerwalt et al. (25) and Brenner (2) more than 30 years ago, it has been recognized that Hafnia alvei, as originally proposed and described, was heterogeneous at the DNA level, consisting of two or more genetically distinct species termed DNA groups or genomospecies. These species could not easily be separated and identified biochemically, although both taxa were distinguishable using techniques such as 16S rRNA gene sequencing (16). The problem of identifying some differential biochemical characteristics has been recently overcome, and Huys and colleagues (13) have proposed a new Hafnia species, H. paralvei, which was previously referred to as H. alvei hybridization (DNA) group 2. Although both H. alvei and H. paralvei have been recovered from clinical samples, little is known regarding their relative frequency or disease distribution. Additionally, it is unclear whether the previously proposed phenotypes used to separate each species are useful in the clinical laboratory. We have studied a large collection of Hafnia isolates to answer the above-described questions and propose some additional new and novel phenotypic and genotypic markers to aid in the recognition of these two species. Finally, we propose one potential virulence characteristic (cytolysin) in specific Hafnia strains which may be associated with certain clinical syndromes (gastrointestinal and related sequelae). These investigations and observations serve as the basis of this report.

MATERIALS AND METHODS

Strains. A total of 68 Hafnia strains were evaluated in this study. These strains were part of the Microbial Diseases Laboratory collection from 1970 to the present. All strains were of clinical origin, and most were isolated from the gastrointestinal tract (n = 39). Miscellaneous clinical sites included the respiratory tract (n = 5), urine (n = 3), a wound (n = 1), and unknown sources (n = 20). Repeated attempts to secure blood isolates for this investigation were unsuccessful. All strains were biochemically identified as Hafnia and were KCN, Voges-Proskauer, and glyceral positive and negative for glutamate decarboxylase activity, which distinguishes this group from Escherichia albertii (15). Each strain was additionally identified to the species level as H. alvei or H. paralvei using 16S rRNA gene sequencing as previously described (16). Some strains were also identified to the species level by DNA-DNA hybridization (13). The type or reference strains used in these studies included H. alvei ATCC 13337T and ATCC 29926 and H. paralvei ATCC 29927T and CCUG 429.

Biochemical tests and antimicrobial susceptibility. Hafnia strains were screened against a standard set of biochemical characteristics previously identified as useful in the separation of H. alvei from H. paralvei. These tests included malonate utilization (48 h), D-arabinose and salicin fermentation (96 h), and β-glucosidase activity (13, 16, 17). β-Glucosidase activity was measured by fluorescence after 5 min of incubation in the dark on bacterial impregnated disks from Key Scientific (Stamford, TX). Antibiotic susceptibility testing was performed for 35 hafniae (H. alvei, n = 16; H. paralvei, n = 19) using Etest (AB Biodisk, Solna, Sweden) according to the manufacturer’s instructions. The panel of antibiotics tested included routine agents used against the family Enterobacteriaceae as listed in Clinical and Laboratory Standards Institute (CLSI) document M100-S20 (6). Colistin resistance was confirmed using the broth microdilution method on a selected number of strains with a MIC of ≥4 μg/ml.
ampC gene sequences. The same 35 Hafnia strains used for susceptibility testing were subjected to partial ampC gene sequence analysis. Consensus primers for ampC amplification were designed using Hafnia ampC cephalosporinase gene sequences available in GenBank. PCR primers AmpCF (5'-GTATCTCCGTGATACCTGTCTGGC) and AmpCR (5'-CTGCCCCATATTGGCTTGAC) were used to amplify an internal portion of the ampC gene (~770-bp product) in all Hafnia strains. The purified PCR product was sequenced using the same primers. Sequence editing, assembly, and analysis were performed using Sequencer version 4.10.1 (Gene Codes Corporation, Ann Arbor, MI). A BLAST (NCBI) nucleotide sequence query was used to infer cephalosporinase gene identity. A phylogeny based on ampC sequences was constructed using the neighbor-joining algorithm of MEGA version 4 (26).

RESULTS

Biochemical studies. We screened a large number of phenotypic properties (>60) for discriminatory value in the separation of H. alvei and H. paralvei. These tests included some phenotypes previously suggested by Brenner (3) as potentially having differential value. In our laboratory, tests such as motility at 24 h, indol, and sodium acetate utilization did not discriminate between the two Hafnia species as previously noted by others (3). Still other potential discriminatory tests that were associated exclusively with only one species, such as raffinose fermentation, were found at such low frequencies to make their usefulness in laboratory identification meaningless (16). However, both malonate utilization and fermentation of D-arabinose, which were previously linked to specific Hafnia DNA groups or biotypes, appeared more useful in species differentiation.

Based upon taxonomic investigations (13) with a limited number of strains, we chose four tests because they are commonly used in the clinical laboratory or easily interpreted and/or the overall expression of the phenotype in hafniae was strong. The results are presented in Table 1. All 68 Hafnia strains could be unambiguously assigned to the correct species based upon this battery of four tests. H. alvei sensu stricto strains were typically malonate negative, while D-arabinose was positive, and D-glucosidase was negative, while the opposite pattern was associated with the newly described species H. paralvei. Among the type or reference strains, ATCC 13337 was atypical in being malonate negative, while ATCC 29927 was phenotypically aberrant in being the only (weakly) D-glucosidase-positive H. paralvei strain. The association of β-glucosidase activity with H. alvei was also confirmed in other aglycone compounds (es-

<table>
<thead>
<tr>
<th>Test</th>
<th>No. of H. alvei isolates</th>
<th>No. (%) positive</th>
<th>No. of H. paralvei isolates</th>
<th>No. (%) positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malonate</td>
<td>13</td>
<td>14</td>
<td>1</td>
<td>27 (96)</td>
</tr>
<tr>
<td>D-Arabinose</td>
<td>3</td>
<td>7</td>
<td>18</td>
<td>10 (36)</td>
</tr>
<tr>
<td>Salicin</td>
<td>18</td>
<td>0</td>
<td>18</td>
<td>18 (64)</td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td>20</td>
<td>8</td>
<td>0</td>
<td>28 (100)</td>
</tr>
</tbody>
</table>

* + +, strong reaction at 24 h; +, delayed or weak reaction; −, negative.
* Total n = 28.
* Total n = 40.

Antimicrobial susceptibility. Regardless of the species designation, all Hafnia strains were uniformly susceptible to amikacin, gentamicin, ciprofloxacin, gatifloxacin, ofloxacin, trimethoprim-sulfamethoxazole, imipenem, meropenem, aztreonam, and ceftizoxime (Table 2). More than half of the strains tested were resistant to tetracycline, with variable susceptibility to chloramphenicol noted. The greatest resistance to antimicrobials within the genus was noted with β-lactams, β-lactam inhibitor combinations, and cephalosporins. There was a significant difference in susceptibility to ampicillin-sulbactam (Fisher’s exact test, P ≤ 0.04) between H. alvei and H. paralvei. H. paralvei isolates were less susceptible to these classes of antibiotics than those of H. alvei were. The majority of the strains were intermediately susceptible or resistant to ceftazidime, where resistance to other extended-spectrum cephalosporins was variable. No cephalosporin susceptibility patterns specific to H. alvei or H. paralvei were identified.
Since there are no published CLSI guidelines for colistin, the European Committee on Antimicrobial Susceptibility Testing and British Society for Antimicrobial Chemotherapy clinical breakpoints for Enterobacteriaceae of $\leq 2$ and $>2 \mu g/ml$ were used (4, 10). Using these breakpoints, 80% of the isolates included in this study would be considered resistant (Fig. 1A). H. paralvei strains were more likely than H. alvei strains to be resistant to colistin (Fisher’s exact test, $P \leq 0.001$). We did not attempt to determine the mechanism of colistin resistance among our isolates but did note that two strains with a MIC of $8 \mu g/ml$ had a visibly rough colonial morphotype. We noted an unusual response in approximately one-third of the Hafnia strains, whereby we observed growth formation into the susceptibility zone along and extending from the increasing antibiotic gradient (Fig. 1B).

AmpC $\beta$-lactamases. Hafnia often exhibits variable resistance to cephalosporins due to the presence of chromosomal AmpC $\beta$-lactamases. These cephalosporinases may display either a low-level inducible or high-level constitutive activity (11, 15, 27). We hypothesized that sufficient divergence of these two Hafnia species may have occurred that they could be distinguished on the basis of ampC gene sequences. A sequence query revealed that all of our H. alvei isolates (16/16) had either the acc-3 gene or the acc-3a variant. Of our H. paralvei strains, 95% (18/19) contained the acc-1 gene or variant acc-1a, acc-1b, acc-1c, or acc-1d ($P < 0.001$). A single H. paralvei strain contained the acc-2 cephalosporinase gene. Tree topology based on analysis of the ampC gene sequence data set separates Hafnia into two clades with 100% correlation to species separation based on previous 16S rRNA gene sequencing (Fig. 2).

Cytolytic activity. In a previous report (7), we described a probable case of HUS in an 11-year-old girl that was associated with the repeated isolation of a toxigenic strain of Hafnia from feces. That strain produced Shiga toxin-like activity in Vero cells that was not neutralized by type 1 or 2 Shiga toxin antibodies. We investigated whether this activity was strain specific or if it might be commonly found in other members of the genus Hafnia. A CPE was observed in 63% of the Hafnia strains (76% of the fecal strains) tested when cell-free supernatants were exposed to Vero cell monolayers (Table 3). Despite the high overall frequency of toxigenic activity detected, differences in toxigenic activity between the two species were noted. H. alvei strains were more likely to be toxigenic (80% versus 47%) and express a stronger CPE (73% versus 6%) than H. paralvei isolates. The first indications of a CPE were observed in all but two strains of H. alvei as early as 48 h even though final results were not recorded until 96 h postinoculation; only one strain of H. paralvei demonstrated a CPE at 48 h. The CPE detected was indistinguishable from that produced by Shiga toxin-producing E. coli O157:H7.

FIG. 1. Colistin susceptibility testing of Hafnia. (A) Distribution of strains by species and MIC. (B) Unusual zone of susceptibility to colistin exhibited by approximately one-third of the hafniae tested.

DISCUSSION

Results from the present investigation indicate that both H. alvei and H. paralvei can be frequently isolated from clinical laboratory samples and that a number of simple and easy-to-perform phenotypic tests are available to aid in their recognition (Table 1). The fact that all of the H. alvei strains tested to date carry either the aac-3 gene or a variant provides yet another differential characteristic at the molecular level to aid in the identification of both species. In an extensive review of clinical reports involving Hafnia infections, we found several cases of bacteremia caused by H. alvei sensu stricto (DNA group 1) because the infecting strain was described as either salicin or esculin positive (9, 18). Unfortunately, it is not possible to make a similar retrospective association for H. paralvei since the significant phenotype (fermentation of $d$-arabinose) is not commonly tested for in microbiology laboratories. However, in a case report from our laboratory describing an apparent association between a toxigenic Hafnia isolate and a case of HUS, the isolate was confirmed to be H. paralvei by biochemical testing and 16S rRNA gene sequencing (7). Skurnik et al. (24) reported on an isolate taken during surgery for a peritoneal infection in a 53-year-old male with multiple medical problems; that strain was also identified as a drug-resistant strain of H. paralvei by 16S and rpoB gene sequencing. However, the role of that strain in disease pathogenesis is unclear.

Although the susceptibility profiles of these Hafnia species did not differ dramatically, some differences were noted (ampicillin-sulbactam) and widespread resistance to colistin was observed. Colistin resistance in Hafnia has been previously reported, but the mechanisms mediating resistance in these cases have yet to be fully determined. In a retrospective study by Günthard and Pennekamp (12), laboratory results of disk

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Diffusion susceptibility testing of 72 H. alvei isolates showed that one-fourth of the strains were resistant to colistin. Colistin-resistant H. alvei has also been isolated from the feces of two leukemic patients suffering from gastrointestinal disease (21). These reports are cause for concern because colistin represents an antibiotic class that is among the limited treatment options for infections caused by extended-spectrum β-lactam- or multidrug-resistant organisms, and Hafnia spp. are often recovered as part of mixed infections. Further characterization of colistin resistance mechanisms in Hafnia spp. is warranted, as these organisms are associated with polymicrobial nosocomial infections.

An interesting observation was the common expression of a cytolytic toxin by strains of H. alvei and H. paralvei on Vero cell monolayers. This cytolytic toxin was found in many Hafnia isolates (Table 3). Very little is known regarding the pathogenicity or virulence characteristics of hafniae, although siderophores, resistance to complement-mediated lysis, and possession of type 1 and 3 fimbriae have been described (19). The intriguing aspect of the present finding is that the toxin is expected to play a significant role in the interactions between Hafnia spp. and their host.

**Table 3.** Cytolytic activities of Hafnia strains on Vero cells

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of strains</th>
<th>CPE on Vero cells</th>
<th>Sources (no. of isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. alvei</td>
<td>15</td>
<td>++++ + + + +</td>
<td>Stool (10), sputum (1), urine (1), unknown clinical (3)</td>
</tr>
<tr>
<td>H. paralvei</td>
<td>17</td>
<td>11 1 3 4 9</td>
<td>Stool (6), sputum (2), urine (2), nasosuction (1), trachea (1), wound (1), unknown clinical (4)</td>
</tr>
</tbody>
</table>

*CPEs: ++++, 100%; ++, 50%; +, 25%; -, negative.*
common in many fecal strains and, in at least one case, linked to bloody diarrhea and HUS (7). Donato et al. (8) have determined that Hafnia strains used to infect polarized MDCK-I and T84 cells decrease transepithelial electrical resistance and increase permeability to a dextran probe, although they do not redistribute tight-junction zona occludens-1 or claudin-1. H. alvei sensu lato has been implicated on several occasions as a cause of bacterial gastroenteritis, and at least two outbreaks of diarrheal disease have been attributed to this organism (15, 20). It has been proposed that “H. alvei” is a candidate enteric pathogen (8), and perhaps this cytolytic toxin plays a role in this process. Further investigations concerning the role that hafniae may play in gastroenteritis seem warranted.

Little is known regarding the frequency, anatomic distribution, and disease spectrum of H. alvei or H. paralvei based upon recent taxonomic proposals (13). The identification of simple phenotypic tests and molecular drug resistance markers should greatly aid in answering these questions. It is tempting to speculate that H. alvei may be more pathogenic than H. paralvei based upon the frequency and intensity of cytolsin production by this species and its known association with published cases of septicemia. However, more case reports and pathogenicity studies using in vitro and in vivo models need to be performed in order to answer these questions.

REFERENCES