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Comparison of Ribotyping and Restriction Enzyme Analysis for Inter- and Intraspecies Discrimination of *Bordetella avium* and *Bordetella hinzii*

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Bordetella avium **is an avian respiratory disease pathogen responsible for substantial economic losses to the turkey industry. The inability to distinguish isolates has hampered outbreak investigations and prevents a complete understanding of transmission mechanisms. Isolates of *Bordetella hinzii*, often referred to as *B. avium*-like or as *Alcaligenes faecalis* type II prior to 1995, have also been acquired from the respiratory tracts of diseased poultry but are not believed to be pathogenic for birds. Therefore, differentiating between *B. avium* and *B. hinzii* is of importance for veterinary diagnostic laboratories. It was recently reported that both *Pvu*II ribotyping and *Hin*fI/*Dde*I restriction endonuclease analysis (REA) show promise for distinguishing isolates of *B. avium* and *B. hinzii*. Here we compare the ability of these techniques to discern inter- and intraspecies differences. While both approaches distinguished numerous types within a species, only REA was sufficiently discriminatory for routine use as an epidemiologic tool. Both techniques clearly distinguish between *B. avium* and *B. hinzii*, although the results of ribotyping are more easily interpreted. Ribotyping and REA identified numerous, previously unrecognized *B. hinzii* strains from a collection of bordetella isolates, including one acquired from a rabbit. This is the first report of *B. hinzii* isolation from a nonhuman mammalian species. At least some of the newly recognized *B. hinzii* isolates have been previously reported to cause disease in poults, suggesting that the pathogenicity of this agent for poultry should be more rigorously examined.**

Bordetella avium is the etiologic agent of coryza or rhinotracheitis in poultry, a highly contagious disease resulting in substantial economic losses to the turkey industry (25). Epidemiologic investigation of outbreaks is not generally undertaken, since no typing system is available for differentiating among isolates. A recent report demonstrated that restriction endonuclease analysis (REA), based on restriction fragment polymorphisms resulting from digestion with *Hin*fI and, separately, with *Dde*I, may be a useful discriminatory tool (23). Data from the same study suggested that ribotyping with *Pvu*II, previously used to distinguish *Bordetella bronchiseptica* isolates (19, 20), might also be employed for categorizing strains of *B. avium*, although only a few isolates were evaluated.

Bordetella hinzii, referred to as *B. avium*-like or as *Alcaligenes faecalis* type II prior to 1995 (27), has also been acquired from the respiratory tracts of diseased poultry but has not been demonstrated to be pathogenic for birds (2, 11, 12). Consequently, differentiating between *B. avium* and *B. hinzii* is of importance for veterinary diagnostic laboratories. Several human isolates have also been reported recently, and most were documented to cause disease, including one fatality (6, 7, 8, 14, 27). Therefore, accurate identification of *B. hinzii* is also of importance for human diagnostic laboratories. A few biochemical tests have been shown to delineate *B. avium* and *B. hinzii*, but they do not correctly identify all strains (27) and results of some may vary depending on inoculum size and the procedure used (1, 3, 9). Although these species have been shown to possess unique cellular fatty acid profiles (6, 7, 13, 27), the results are affected by culture conditions (14, 27). Discriminatory methods based on stable genetic elements, rather than variable phenotypic characteristics, are likely to provide a more reliable approach. Both REA analysis, using either *Hin*fI or *Dde*I (23), and 16S rRNA sequence analysis (14) have been shown to differentiate between *B. avium* and *B. hinzii*. Preliminary results, based on a comparison of the patterns obtained from seven *B. avium* isolates and eight *B. hinzii* isolates, indicate that *Pvu*II ribotyping may likewise be useful for identification of these species (23). A comparison of ribotyping and REA for typing isolates of *B. hinzii* is also of interest, given the increasing importance of this organism as an opportunistic human pathogen.

In the present study, 57 *B. avium* isolates, a *B. avium* vaccine strain, and 11 *B. hinzii* isolates were evaluated by *Pvu*II ribotyping to more fully assess the discriminatory capacity of this method for distinguishing these two species. Additionally, 5 isolates classified as *B. avium*-like, 18 isolates identified as *A. faecalis* type II prior to the establishment of *B. hinzii* as a species, and 5 isolates previously identified as atypical *B. bronchiseptica* were analyzed by REA and ribotyping to ascertain their proper classification. The discriminatory power of REA and ribotyping for intraspecies classification was quantitated to determine the suitability of these methods as epidemiologic tools.

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MATERIALS AND METHODS

Bacterial isolates and growth conditions. The isolates studied are listed in Table 1 according to their original designations. Isolates with the prefix DBL

TABLE 1. *B. avium* isolates evaluated in this study

Species	Strain	Yr of isolation[b]	Geographic origin	Host	Ribotype[a]	Profile	
						*Hin*fI[a]	*Dde*I[a]
B. avium	ATCC 35086[T]	1977	Germany	Turkey	BA5	BA007	BA002
B. avium	197N	1983	Ohio	Turkey	BA1	BA002	BA005
B. avium	4084	1979 or prior	North Carolina	Turkey	BA1	BA001	BA001
B. avium	4085	1979 or prior	North Carolina	Turkey	BA2	BA006	BA010
B. avium	4087	1979 or prior	North Carolina	Turkey	BA4	BA002	BA001
B. avium	4089	1979 or prior	Germany	Turkey	BA5	BA007	BA002
B. avium	4091	1979 or prior	Germany	Turkey	BA1	BA001	BA001
B. avium	4092	1979 or prior	Germany	Turkey	BA1	BA001	BA001
B. avium	4093	1979 or prior	Germany	Turkey	BA6	BA007	BA003
B. avium	4094	1979 or prior	Germany	Turkey	BA6	BA007	BA003
B. avium	4095	1979 or prior	North Carolina	Turkey	BA1	BA003	BA005
B. avium	4139	1979 or prior	Minnesota	Turkey	BA1	BA001	BA011
B. avium	4142	1979 or prior	Minnesota	Turkey	BA1	BA001	BA001
B. avium	4143	1979 or prior	Iowa	Turkey	BA1	BA003	BA005
B. avium	4148	1979 or prior	Ohio	Turkey	BA1	BA001	BA004
B. avium	4149	1979 or prior	Ohio	Turkey	BA1	BA001	BA001
B. avium	4150	1979 or prior	Ohio	Turkey	BA1	BA001	BA001
B. avium	4151	1979 or prior	Ohio	Turkey	BA1	BA001	BA001
B. avium	4152	1979 or prior	Ohio	Turkey	BA1	BA001	BA004
B. avium	4153	1979 or prior	Ohio	Turkey	BA1	BA001	BA001
B. avium	4154	1979 or prior	Ohio	Turkey	BA1	BA001	BA001
B. avium	4155	1979 or prior	Ohio	Turkey	BA1	BA001	BA001
B. avium	4156	1979 or prior	Ohio	Turkey	BA1	BA001	BA001
B. avium	4157	1979 or prior	Ohio	Turkey	BA1	BA001	BA001
B. avium	4158	1979 or prior	Ohio	Turkey	BA1	BA001	BA001
B. avium	4163	1979 or prior	Ohio	Turkey	BA1	BA002	BA001
B. avium	4164	1979 or prior	Ohio	Turkey	BA1	BA002	BA001
B. avium	4165	1979 or prior	Ohio	Turkey	BA1	BA002	BA001
B. avium	4166	1979 or prior	Ohio	Turkey	BA1	BA002	BA001
B. avium	4167	1979 or prior	Ohio	Turkey	BA1	BA002	BA001
B. avium	4168	1979 or prior	Ohio	Turkey	BA1	BA002	BA001
B. avium	4169	1979 or prior	Ohio	Turkey	BA1	BA001	BA001
B. avium	4480	1981 or prior	Iowa	Turkey	BA1	BA008	BA012
B. avium	4481	1981 or prior	Iowa	Turkey	BA1	BA002	BA001
B. avium	4482	1981 or prior	Iowa	Turkey	BA1	BA001	BA008
B. avium	4483	1981 or prior	Iowa	Turkey	BA1	BA001	BA009
B. avium	4484	1981 or prior	Iowa	Turkey	BA1	BA002	BA001
B. avium	4485	1981 or prior	Iowa	Turkey	BA1	BA002	BA005
B. avium	4486	1981 or prior	Iowa	Turkey	BA1	BA003	BA005
B. avium	4506	1981 or prior	South Africa	Turkey	BA3	BA004	BA007
B. avium	4507	1981 or prior	South Africa	Turkey	BA3	BA001	BA006
B. avium	4508	1981 or prior	South Africa	Turkey	BA3	BA004	BA006
B. avium	T4	1998	New Jersey	Turkey	BA1	BA011	NT[c]
B. avium	D4	1998	New Jersey	Duck	BA1	BA007	NT
B. avium	D10	1998	New Jersey	Duck	BA3	BA009	NT
B. avium	D23	1998	New Jersey	Duck	BA3	BA009	NT
B. avium	D24	1998	New Jersey	Duck	BA3	BA009	NT
B. avium	D25	1998	New Jersey	Duck	BA3	BA009	NT
B. avium	D26	1998	New Jersey	Duck	BA3	BA009	NT
B. avium	D27	1998	New Jersey	Duck	BA3	BA009	NT
B. avium	G24	1998	New Jersey	Goose	BA7	BA012	BA014
B. avium	DBL-239	1997	Minnesota	Turkey	BA1	BA014	BA004
B. avium	DBL-254-1	1997	Minnesota	Turkey	BA1	BA010	BA014
B. avium	DBL-260	1997	Minnesota	Turkey	BA8	BA010	BA004
B. avium	DBL-971	1997	Minnesota	Turkey	BA1	BA010	BA004
B. avium	DBL-O1067	2001	Iowa	Turkey	BA1	BA010	BA011
B. avium	DBL-191	1989	New York	Turkey	BH2	BH008	BH002
B. avium	Art-Vax	Prior to 1980	North Carolina	Turkey	BA1	BA005	BA001
B. avium-like	DBL-019	2000	Pennsylvania	Turkey	UK[d]	UK[d]	UK[e]
B. avium-like	DBL-245	1997	Minnesota	Turkey	BA1	BA013	BA015
B. avium-like	DBL-254-3	1997	Minnesota	Turkey	BA1	BA004	BA013
B. avium-like	DBL-243-2	1997	Minnesota	Turkey	BH2	BH004	NT
B. avium-like	GOBL110	1985 or prior	United States	Turkey	BH2	BH009	BH001
B. hinzii	L60 (ATCC 51730)	1994	Washington	Human	BH1	BH003	BH001
B. hinzii	ATCC 51783[T]	UK	Australia	Chicken	BH1	BH003	BH001

Continued on following page

TABLE 1—*Continued*

Species	Strain	Yr of isolation[b]	Geographic origin	Host	Ribotype[a]	Profile	
						*Hin*fI[a]	*Dde*I[a]
B. hinzii	ATCC 51784	UK	Belgium	Chicken	BH2	BH004	NT
B. hinzii	1277	1992	Switzerland	Human	BH1	BH006	BH004
B. hinzii	1280	1993	Switzerland	Human	BH1	BH006	BH004
B. hinzii	96025473	1999	Spain	Human	BH1	ND[f]	ND
B. hinzii	4134	1979 or prior	Minnesota	Turkey	BH1	BH003	BH001
B. hinzii	4159	1979 or prior	Ohio	Turkey	BH1	BH003	BH001
B. hinzii	4161	1979 or prior	Ohio	Turkey	BH1	BH003	BH001
B. hinzii	4509	1981 or prior	South Africa	Chicken	BH2	BH004	BH001
B. hinzii	TR96-1212	1996	United States	Turkey	BH1	BH005	BH001
A. faecalis type II	4137	1979 or prior	Minnesota	Turkey	UK[d]	UK[d]	UK[d]
A. faecalis type II	4138	1979 or prior	Minnesota	Turkey	UK[d]	UK[d]	UK[d]
A. faecalis type II	4140	1979 or prior	Minnesota	Turkey	BH1	BH005	BH003
A. faecalis type II	4141	1979 or prior	Minnesota	Turkey	BH1	BH005	BH003
A. faecalis type II	4147	1979 or prior	Ohio	Turkey	BH1	BH004	BH001
A. faecalis type II	4160	1979 or prior	Ohio	Turkey	BH1	BH002	BH002
A. faecalis type II	4162	1979 or prior	Ohio	Turkey	BH1	BH002	BH002
A. faecalis type II	4445	1981 or prior	Minnesota	Turkey	BH1	BH005	BH001
A. faecalis type II	4449	1981 or prior	Minnesota	Turkey	BH3	BH007	BH001
A. faecalis type II	4595	1982 or prior	Iowa	Turkey	BH1	BH003	BH001
A. faecalis type II	4596	1982 or prior	Iowa	Turkey	BB15	BB010	ND
A. faecalis type II	4597	1982 or prior	Iowa	Turkey	BH2	BH004	BH001
A. faecalis type II	4598	1982 or prior	Iowa	Turkey	BH2	BH004	BH006
A. faecalis type II	4599	1982 or prior	Iowa	Turkey	BH2	BH004	BH005
A. faecalis type II	4081	1979 or prior	North Carolina	Turkey	UK[d]	UK[d]	UK[e]
A. faecalis type II	4086	1979 or prior	North Carolina	Turkey	UK[d]	UK[d]	UK[d]
A. faecalis type II	4135	1979 or prior	Minnesota	Turkey	BH1	BH001	BH001
A. faecalis type II	4136	1979 or prior	Minnesota	Turkey	BH3	BH007	BH001
B. bronchiseptica	4444	1981 or prior	Minnesota	Turkey	UK[d]	UK[d]	UK[e]
B. bronchiseptica	4447	1981 or prior	Minnesota	Turkey	BH3	BH004	BH001
B. bronchiseptica	4450	1981 or prior	Minnesota	Turkey	BH3	BH007	BH001
B. bronchiseptica	4451	1981 or prior	Minnesota	Turkey	BH2	BH004	BH001
B. bronchiseptica	5132	1990	Hungary	Rabbit	BH2	BH004	BH001

[a] *B. avium* ribotypes and REA patterns have the prefix BA, *B. hinzii* ribotypes and REA patterns have the prefix BH, and *B. bronchiseptica* ribotypes and REA patterns have the prefix BB.
[b] Where a year is followed by "or prior," isolates were received at the National Animal Disease Center during the year indicated; the actual year of isolation is unknown.
[c] NT, not typeable.
[d] Unknown; pattern does not match or cluster within *B. avium*, *B. hinzii*, or *B. bronchiseptica* profiles.
[e] Unknown; pattern does not match, but does cluster within, previously identified *B. avium* profiles.
[f] ND, not determined.

were kindly provided by Linda Schroeder-Tucker, National Veterinary Services Laboratories, Diagnostic Bacteriology Laboratory, Ames, Iowa. *B. avium* 197N and *B. avium*-like isolate GOBL110 (provided by Louise Temple, Drew University, Madison, N.J.) were originally acquired by the laboratory of Y. M. Saif, The Ohio State University, Wooster. The *B. avium* vaccine strain Art-VaxJ (4) (Schering-Plough Animal Health, Union, N.J.) was purchased from a commercial vendor. Other isolates generously provided by colleagues include *B. hinzii* DMMZ 1277 and DMMZ 1280 (7) (Reinhard Zbinden, Institute for Medical Microbiology, Zurich, Switzerland), *B. hinzii* 96025473 (8) (Ignacio Gadea, Department of Medical Microbiology, Fundación Jiménez Díaz, Madrid, Spain), *B. bronchiseptica* 5132 (Tibor Magyar, Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest), and *B. hinzii* TR96-1212 (Louise Temple, Drew University). All remaining isolates were obtained from a collection at the National Animal Disease Center. Bacteria were grown on sheep's blood agar at 37°C for 18 to 36 h.

Ribotyping. Genomic DNA was isolated using a commercially available kit (Gentra Systems, Minneapolis, Minn.). Ribotyping analysis was based on hybridization of *Pvu*II digestion fragments with a portion of the *Escherichia coli* rRNA operon *rrnB* followed by chemiluminescent detection, as described previously (19, 20). The ribotypes of six *B. avium* isolates included in this study have been previously reported (17).

REA. DNA isolation and REA were carried out as previously described (23). Briefly, bacterial cells were harvested in 0.85 M NaCl, pelleted by centrifugation and stored at −70°C. DNA was isolated using a commercially available kit (DNAzol; Gibco-BRL, Gaithersburg, Md.) according to recommendations of the manufacturer. Fragments resulting from digestion with *Hin*fI or *Dde*I were separated by electrophoresis in 0.7% agarose gels using TBE buffer (0.089 M Tris, 0.089 M boric acid, 2 mM EDTA, pH 8.0). Gels were stained with ethidium bromide and photographed. Profiles of some isolates included in this study have been previously determined (23).

Data analysis. Photographs or chemilumigraphs were scanned for computer analysis using a ScanJet IIcx with DeskScan software (Hewlett-Packard, Boise, Idaho). GelCompar software (Applied Maths, Kortrijk, Belgium) was used for comparison of fingerprint profiles. Ribotypes are designated by Arabic numerals, with *B. avium* types given the prefix BA, *B. hinzii* ribotypes given the prefix BH, and *B. bronchiseptica* types given the prefix BB. REA profiles are designated using the previously proposed DIE code (23), based on three-place Arabic numerals preceded by BA, BH, or BB, as appropriate. Strains that exhibited single-band differences were assigned to different ribotypes or REA profiles. Similarity between all possible pairs of ribotypes or REA profiles using the coefficient of Dice (26) was calculated by the cluster analysis module of the software. Dendrograms were derived from a matrix of similarity values by the unweighted pair group method using arithmetic averages, based on a tolerance of 0.5% for ribotyping and 1.0% for REA. The discriminatory power of ribotyping and/or REA analysis was defined by calculating discrimination indices as described (10).

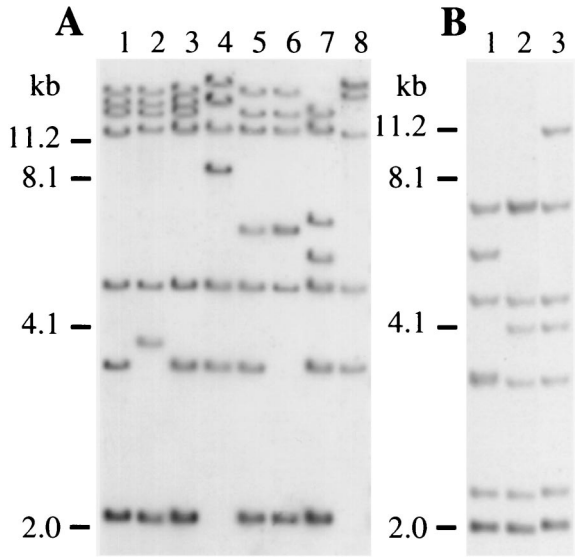

FIG. 1. Ribotype patterns observed following digestion of *B. avium* (A) or *B. hinzii* (B) genomic DNA with *Pvu*II. Lane numbers correspond to the assigned ribotype profile.

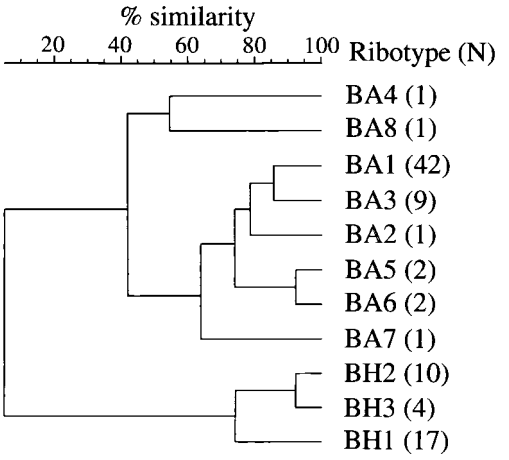

FIG. 2. Dendrogram and cluster analysis of the *B. avium* and *B. hinzii* *Pvu*II ribotype patterns identified from isolates in Table 1 (tolerance, 0.5%). *B. avium* patterns are assigned the prefix BA; *B. hinzii* patterns are assigned the prefix BH. The number of isolates identified for each ribotype is indicated in parentheses to its right.

RESULTS

Ribotyping. The ribotype of individual isolates is indicated in Table 1. A total of nine unique patterns was observed among the *B. avium* isolates examined based on combinations of 15 fragments ranging in size from approximately 2 to 16 kb. Four patterns were identical to previously reported *B. avium* ribotypes (17, 23) (Fig. 1A, lanes 1, 2, 3, and 7), while four others constitute novel types (Fig. 1A, lanes 4, 5, 6, and 8). The ninth pattern, associated only with isolate DBL-191, does not display the cluster of bands greater than 11 kb characteristic of *B. avium* isolates and shares only 5.9% similarity with the remaining eight *B. avium* profiles. This pattern was later found to be identical to one associated with some isolates of *B. hinzii* (Fig. 1B, lane 2). Accordingly, isolate DBL-191 was reclassified as *B. hinzii*. Similarity among the 8 true *B. avium* profiles, designated BA1 to BA8, ranges from 41.9 to 92.3% (Fig. 2).

Two ribotype patterns, designated BH1 and BH2, were identified from the 11 *B. hinzii* isolates analyzed based on eight fragments of roughly 1.8 to 6 kb (Table 1; Fig. 1B, lanes 1 and 2). These profiles share only 5% similarity with *B. avium* profiles and are immediately recognizable as unique by visual comparison (Fig. 1 and 2). BH1 and BH2 profiles have a calculated similarity of 74.2% (Fig. 2).

Since ribotyping readily distinguishes between *B. avium* and *B. hinzii*, this technique was used to evaluate 5 isolates characterized as *B. avium*-like and 18 previously identified as *A. faecalis* type II (22). Recent research suggests that isolates in these categories are more properly classified as *B. hinzii* (27). Thirteen of the 23 isolates displayed either BH1 or BH2 profiles. Two isolates have a profile with 92.3% similarity to BH2, which was designated BH3 (Fig. 1B, lane 3; Fig. 2). Of the remaining eight isolates examined, two possess BA1 ribotypes, one has a *B. bronchiseptica* ribotype 15 profile (19, 20), and five displayed patterns that bear no resemblance to previously defined *B. avium*, *B. hinzii*, or *B. bronchiseptica* patterns. However, the profile of one (strain 4081) is nearly identical to the pattern of fragments observed for *A. faecalis* strain 4615, obtained from the Centers for Disease Control and Prevention (K. B. Register, unpublished data).

These results, combined with previous analysis of over 250 *B. bronchiseptica* isolates (19, 20, 21), demonstrate that ribotype profiles of *B. avium*, *B. hinzii*, and *B. bronchiseptica* are readily distinguishable from one another. Therefore, this technique was used to evaluate a group of five isolates originally identified by other laboratories as *B. bronchiseptica* but which were found in our laboratory to have one or more atypical phenotypic characteristics (Table 1). These isolates were acquired prior to the description of *B. hinzii* as a species. Four isolates displayed *B. hinzii* ribotype profiles. The profile of isolate 4444 did not resemble those of either *B. avium*, *B. hinzii*, or *B. bronchiseptica* and was also unique compared to the *A. faecalis* profile observed above.

Distribution of ribotypes. *B. avium* ribotypes BA1 and BA3 are the most prevalent among the isolates examined here (71 and 15%, respectively) and are also among the most closely related, with 85.7% similarity (Fig. 2). No geographic or chronologic association was evident for these isolates. They were acquired from several different regions of the United States or from Germany during a period of at least 22 years, extending through 2001. The remaining *B. avium* ribotypes are represented by only one or two. The single goose isolate evaluated is the only type BA7 identified and shares the lowest degree of relatedness to other ribotypes in its cluster. Types BA4 and BA8 comprise a separate cluster but are not obviously related to one another based on geographic origin or date of isolation.

Approximately 54.8% of all *B. hinzii* isolates displayed a BH1 profile, while 32.2% were identified as BH2 and 12.9% were identified as BH3. Animal hosts from which *B. hinzii* isolates were identified include turkey, chicken, human and rabbit. The distribution of ribotype profiles among host species is shown in Table 2. The two major *B. hinzii* clusters include isolates from different geographic locations representing four

TABLE 2. *B. hinzii* ribotype frequency and distribution within host species

Ribotype	No. of strains (%) from:			
	Turkey	Chicken	Human	Rabbit
BH1	12 (52.2)	1 (33.3)	4 (100)	1 (100)
BH2	7 (30.4)	2 (66.7)		
BH3	4 (17.4)			
Total	23	3	4	1

continents. Although most isolates were obtained at least 20 years ago, more recent isolates are represented in both clusters. The BH3 profile was found only in isolates acquired in 1981 or earlier. However, this observation may simply reflect the limited number of more recent isolates available for analysis and the lower frequency with which BH3 strains appear to occur. All mammalian isolates have a BH1 profile, but this profile is also frequently found in avian isolates.

REA. *Hin*fI and *Dde*I REA was carried out on all isolates evaluated by ribotyping. Profiles are indicated in Table 1. As previously reported using a smaller group of isolates, REA with either *Hin*fI or *Dde*I clearly discriminated between *B. avium* and *B. hinzii* (Fig. 3). Depending on the enzyme used, REA fingerprints share 8.5 to 17.1% interspecies similarity. Eight *B. avium* isolates and two *B. hinzii* isolates could not be typed using *Dde*I, since the fragments generated were too large to be resolved under the conditions used.

All isolates which displayed *B. avium* ribotypes, including those not originally identified as *B. avium* using biochemical methods, displayed *B. avium*-specific REA patterns. Among these isolates, 14 unique *Hin*fI profiles (Fig. 3A) and 15 unique *Dde*I profiles (Fig. 3B) could be distinguished. *Hin*fI profiles BA009 to BA014 and *Dde*I profiles BA013 to BA015 have not been previously reported and are associated only with isolates acquired since 1997. Depending on the enzyme used, *B. avium* profiles share a minimum of 60.5 to 42.6% similarity. The majority of isolates (52.5%) was represented either by *Hin*fI profile BA001 or BA002. Other *Hin*fI profiles each comprised 1.7 to 10.2% of the remaining isolates. The most common *B. avium Dde*I profile was BA001 (47.0%), with the remaining *Dde*I profiles representing 2.0 to 9.8% of isolates. Twenty-three of the 31 isolates in the two most common *Hin*fI groups also displayed the most common *Dde*I pattern. Identification of less prevalent *Dde*I profiles from the remaining eight isolates demonstrates that additional discriminatory benefit is achieved when analysis is carried out with both enzymes. *Dde*I REA profiles also further distinguished *B. avium* isolates with *Hin*fI BA004, BA007, and BA010 profiles (Table 1).

B. hinzii-specific REA patterns were observed for all isolates previously shown to possess *B. hinzii* ribotypes. Nine *Hin*fI and six *Dde*I profiles were identified from these isolates (Fig. 3). *Hin*fI profiles BH008 and BH009 and *Dde*I profiles BH005 and BH006 have not been previously reported. B. *hinzii* REA patterns share a similarity of at least 81.5% (for *Dde*I) to 84.2% (for *Hin*fI). The most common *B. hinzii Hin*fI profiles are BH004 and BH003, comprising 33.3 and 20.0% of all isolates, respectively. The remaining seven profiles each account for 3.3 to 13.3% of isolates. The *Dde*I pattern associated with the majority of *B. hinzii* isolates is BH001 (67.9%). Other *Dde*I patterns account for 3.6 to 10.7% of isolates. As noted for *B. avium*, nearly all *B. hinzii* isolates in the most common *Hin*fI groups were also members of the most common *Dde*I group. However, a few isolates could be further distinguished when results with both enzymes were considered.

The six isolates originally identified as *B. avium*-like, *A. faecalis* type II, or *B. bronchiseptica* but found not to possess bordetella-specific ribotypes were also evaluated by REA. *Hin*fI and *Dde*I patterns of isolates 4137, 4138, and 4086 do not match or cluster within the group of patterns associated with either *B. avium* or *B. hinzii* (Register, unpublished data). Patterns of isolates DBL-019, 4081, and 4444, all unique from one another, fall within the *B. avium* cluster when results with *Dde*I are considered (Register, unpublished data). However, their *Hin*fI profiles do not match or cluster within either *B. avium* or *B. hinzii* patterns, nor do they cluster with the 48 *Hin*fI patterns previously described for *B. bronchiseptica* (24). Thus, the sum of evidence indicates none of these six isolates are *B. avium*, *B. hinzii*, or *B. bronchiseptica*, and their identities remain unknown.

Discriminatory power of ribotyping and REA. Discrimination indices were calculated for *Pvu*II ribotyping, REA using *Hin*fI, and REA using *Dde*I, both separately and in combination, for *B. avium* isolates and *B. hinzii* isolates (Table 3). A method is generally considered sufficiently discriminatory for typing purposes if an index of 0.900 or greater is obtained (10). No single analysis evaluated here appears sufficiently discriminatory for routine use as a typing tool. However, combined application of *Hin*fI and *Dde*I REA does yield a level of confidence suitable for routine use in epidemiologic investigations. The discriminatory power is slightly increased when ribotyping results are also considered. Two *B. avium* REA types, *Hin*fI BA002-*Dde*IBA001 and *Hin*fI BA010-*Dde*IBA004, could be further distinguished with ribotyping. Isolates with the *B. hinzii* fingerprint profile *Hin*fI BH004-*Dde*IBH001were comprised of three different ribotypes.

DISCUSSION

The results reported here establish the validity of combined *Hin*fI/*Dde*I REA as an epidemiologic tool for both *B. avium* and *B. hinzii*, as proposed in an earlier preliminary study (23). Although the discrimination index associated with *Hin*fI REA alone falls just short of the recommended limit, it may be advantageous to adopt this method as a preliminary tool in outbreak investigations since some isolates cannot be typed by *Dde*I REA and the additional discriminatory power provided is relatively low. While the highest level of discrimination is achieved using a combination of ribotyping and REA, in most cases the modest increase over REA alone is unlikely to justify the added time and expense.

In some instances, isolates that share a high degree of similarity by ribotyping also cluster tightly based on REA. However, in many cases there is no concurrence between ribotype and REA clustering patterns of individual strains. This observation is not surprising, considering that REA is a whole-genome method while ribotyping specifically targets rRNA genes. The superior discriminatory power of REA justifies its use in monitoring the spread of strains during outbreaks.

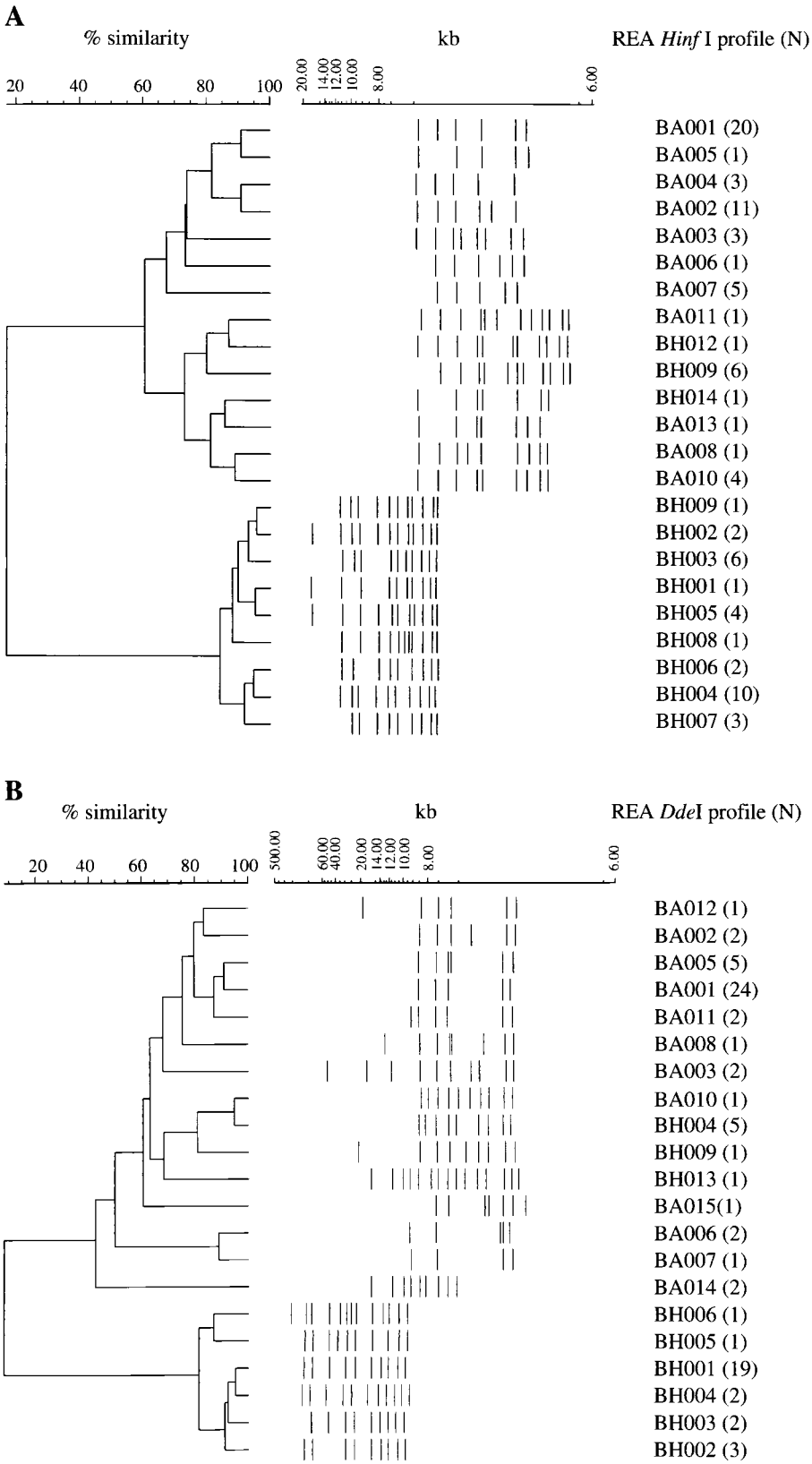

FIG. 3. Dendrogram and cluster analysis of the *B. avium* and *B. hinzii Hin*fI (A) or *Dde*I (B) REA patterns identified from isolates in Table 1 (tolerance, 1%). Individual REA patterns are represented graphically to the right of each dendrogram. *B. avium* patterns are assigned the prefix BA; *B. hinzii* patterns are assigned the prefix BH. The number of isolates identified for each REA pattern is indicated in parentheses to its right.

TABLE 3. Discrimination indices associated with single and combined typing methods

Species	Discrimination index obtained by indicated method(s)				
	RT[a]	REA			RT + REA *Hin*fI/*Dde*I
		*Hin*fI	*Dde*I	*Hin*fI/*Dde*I	
B. avium	0.474	0.835	0.764	0.894	0.901
B. hinzii	0.598	0.837	0.534	0.902	0.926

[a] RT, ribotyping.

Nonetheless, the phenotypic implications of base pair changes resulting in distinct patterns are not clear and may be relatively minor. Ribotyping may be a more accurate indicator of meaningful phylogenetic relationships, given the high degree of conservation found among rRNA genes. Overall, both REA and ribotyping indicate greater genetic diversity among *B. avium* isolates than among *B. hinzii* isolates, suggesting *B. hinzii* may have evolved more recently than *B. avium*. Although *B. hinzii* has only been formally recognized as a unique species since 1995, our data confirm its existence since at least the late 1970s. It is currently unclear whether *B. hinzii* truly has a more recent origin than *B. avium* or whether difficulties related to accurate identification precluded awareness of its existence.

REA results for *B. avium* suggest new variants may have recently emerged. *Hin*fI profiles BA009 to BA014, which cluster together in one of the two major *B. avium Hin*fI groups, are associated exclusively with isolates acquired since 1997. None of the 43 isolates obtained in 1983 or earlier fall within this cluster. The only additional strain in this group was isolated during the interim, in 1989. The majority of the most recent isolates are either nontypeable using *Dde*I, which was not the case for any pre-1997 isolate, or have profiles BA013 to BA015, also not found in older isolates. The *Dde*I dendrogram shows profiles BA013 to BA015 to be the least similar to other profiles in their respective clusters. Analysis of a larger group of recent isolates is needed to better assess the degree of genetic divergence actually occurring. There is strong evidence suggestive of vaccine-driven evolution in the human pathogen *Bordetella pertussis* (15, 16, 28). It has been proposed that clonal expansion of strains began occurring within 10 to 20 years after the initiation of mass vaccination (28). Since widespread vaccination against *B. avium* in poultry has only been implemented in the previous 15 to 20 years, it is tempting to speculate about its potential contribution to the emergence of new variants. Interestingly, REA results indicate the vaccine strain included in this study is among those isolates sharing the least similarity with those obtained recently.

B. hinzii colonizes the respiratory tract of poultry but is generally believed to be nonpathogenic. Thus, it is of importance for veterinary diagnostic laboratories to distinguish this bacterium from *B. avium*, which causes significant losses in the poultry industry. Previous reports have indicated that most *B. avium* isolates agglutinate guinea pig erythrocytes, while *A. faecalis* type II and *B. avium*-like isolates, most of which now appear more properly classified as *B. hinzii*, are hemagglutination negative (12, 25). However, some strains display weak reactions, and the results are not always consistent. We have likewise observed inconsistent results for hemagglutination of sheep and turkey erythrocytes by *B. avium* and *B. hinzii* and found that the outcome is affected by the method used (Register, unpublished data). Most *B. hinzii* isolates are weakly positive when a 96-well plate procedure is employed (18) but negative when tested by a slide agglutination method (22). Currently, there is no readily available technique for reliably distinguishing between *B. avium* and *B. hinzii*. The results reported here demonstrate that both ribotyping and REA are equally useful for this purpose. Ribotyping may be preferable since a quick visual examination of the resulting patterns is the only postassay analysis needed. In contrast, the complexity of REA patterns requires computer-assisted analysis for interpretation. 16S rRNA gene sequencing has recently been proposed as a method for definitive identification of *B. hinzii* (14). Only a single *B. avium* isolate was included in that study, but the numerous base pair differences found between *B. avium* and *B. hinzii* suggest this technique would also detect other *B. avium* isolates, although this has yet to be demonstrated. While 16S rRNA sequencing is a high-resolution technique, it requires amplification of genomic DNA and takes considerably longer to complete than ribotyping. No *Pvu*II sites are present in the *B. avium* and *B. hinzii* 16S rRNA sequences reported, indicating that the sequence variability responsible for distinguishing these species by ribotyping must be located in other regions of the *rrnB* operon included in the probe.

Since the establishment of *B. hinzii* as a separate taxon, relatively few isolates have been reported, and all were derived either from humans or poultry (5–8, 14, 23, 27). Additional, but unrecognized, isolations are likely to have been made by some laboratories, as we discovered numerous additional isolates in our own collection following ribotyping and REA. These were previously identified as *B. avium*-like or misidentified as *B. avium*, *B. bronchiseptica*, or *A. faecalis* type II. Included among these is a rabbit isolate representing the first *B. hinzii* strain found in a nonhuman mammal. The difficulty of distinguishing *B. hinzii* from closely related bacteria may have prevented the discovery of other rabbit isolates, as well as isolates from additional hosts, in the past. Whether *B. hinzii* is pathogenic in rabbits is unclear, since no further information is available regarding the host animal.

The findings presented here suggest that the pathogenicity of *B. hinzii* for poultry should be more rigorously examined. Studies from other laboratories, using isolates described at the time as *B. avium*-like, failed to show virulence in poults (2, 12). More recent attempts to induce disease in 1-day-old chickens and turkey poults with *B. hinzii* have also failed (27). However, at least three isolates (4147, 4159, and 4161), formerly classified as *A. faecalis* type II based on biochemical testing, have been previously reported to cause 100% morbidity in poults with a severity indistinguishable from that of *B. avium* (22). Furthermore, the *B. hinzii* isolate DBL-191, originally misidentified as *B. avium*, is believed to have been the cause of respiratory disease in the flock from which it was obtained. Nonetheless, it is apparent that several other isolates identified in this report as *B. hinzii* are incapable of causing disease in poultry (22). Potential virulence factors of this bacterium have not yet been described. Additional studies may reveal particular bacterial products, produced by only a subset of isolates, that are required for virulence in poultry and/or other hosts.

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