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# Presence of Copper- and Zinc-Containing Superoxide Dismutase in Commensal *Haemophilus haemolyticus* Isolates Can Be Used as a Marker To Discriminate Them from Nontypeable *H. influenzae* Isolates▽

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**Respiratory isolates of *Haemophilus haemolyticus* are regularly misclassified as nontypeable (NT) *Haemophilus influenzae* due to an aberrant hemolytic reaction on blood agar, with implications for treatment. The presence of *sodC* or its cognate protein, copper-zinc superoxide dismutase, can distinguish respiratory isolates of *H. haemolyticus* from NT *H. influenzae* with 100% accuracy.**

Infection with nontypeable (NT) *Haemophilus influenzae* is the most common cause of exacerbations of chronic obstructive pulmonary disease (COPD), characterized by the increased production of mucopurulent sputum and airway obstruction, and is implicated in disease progression (25). The mechanisms underlying the association between NT *H. influenzae* acquisition and exacerbations are poorly understood (5, 18). In a prospective study of COPD patients, Murphy et al. (19) noted that selected sputum isolates of apparent NT *H. influenzae* had an altered phenotype, including slower growth in broth culture and a tendency to form aggregates. On the basis of genetic analyses (ribosomal DNA sequencing, multilocus sequence typing, DNA-DNA hybridization, and sequencing of the gene encoding the outer membrane protein P6), the variant isolates were proven to be *H. haemolyticus*, a bacterium that had not previously been considered a constituent of the respiratory tract flora. By conventional criteria, 102 of 258 (39.5%) and 12 of 44 (27.3%) sputum and nasopharyngeal isolates, respectively, had been misclassified (19). In routine analysis in the clinical laboratory, hemolysis on blood agar is the only characteristic that distinguishes NT *H. influenzae* from *H. haemolyticus* (4, 9, 10). NT *H. influenzae* is the most common bacterial cause of exacerbations of COPD, whereas *H. haemolyticus* is a commensal and does not cause exacerbations. Because most authorities recommend antibiotic therapy for moderate to severe exacerbations of COPD, a rapid and accurate test that discriminates between *H. influenzae* and *H. haemolyticus* will lead to more rational antibiotic therapy for adults with COPD and reduce the unnecessary use of antibiotics. In the work described here, we demonstrate that the presence of *sodC* (or the copper- and zinc-cofactored superoxide dismutase [CuZnSOD] that it encodes) can reliably distinguish *H. haemolyticus* from NT *H. influenzae*.

Bacterial CuZnSODs are enzymes found in the periplasms of various gram-negative bacteria and, in the case of pathogens, are thought to have a role in the protection of organisms against host defense-derived free-radical-mediated damage (1, 2, 6, 11, 12, 15, 27). In extensive studies, we have established that capsulated or noncapsulated *H. influenzae* sensu stricto strains do not produce active CuZnSOD (11, 12, 14, 16). The population of capsulated *H. influenzae* strains falls into two widely separated phylogenetic divisions, and while strains segregating to phylogenetic division II (some serotype a and b strains and serotype f strains) plus serotype e strains (distantly related to phylogenetic division II) possess the *sodC* gene, they do not produce active enzyme (11, 16), probably due to a mutation that converts an active-site histidine to tyrosine (11). Among a collection of 45 NT *H. influenzae* isolates (20), characterized by multilocus enzyme electrophoresis, comprising electrophoretic types 11 to 13, 26 to 27, 29 to 32, 35 to 45, 49 to 51, 53 to 55, 57 to 61, and 63 to 76, neither the *sodC* gene nor the CuZnSOD protein was detected (16). The only NT *H. influenzae* strains that have been shown to produce CuZnSOD are the cryptic genospecies biotype IV strains responsible for urogenital and neonatal infections (16). The cryptic biotype IV strains are genetically distinct, although they are related to *H. influenzae* and *H. haemolyticus* (21–23); but they have not been isolated from the respiratory tract. The cryptic genospecies biotype IV strains can be distinguished from *H. haemolyticus* by a positive ornithine decarboxylase reaction. In a previous study we described the presence of a *sodC* gene and active CuZnSOD in a single strain of *H. haemolyticus* (14). We now hypothesize that the presence of the *sodC* gene and/or CuZnSOD could differentiate *H. haemolyticus* from NT *H. influenzae* isolates obtained from the respiratory tract.

Initially, a collection of NT *H. influenzae* ($n = 10$) and *H. haemolyticus* ($n = 10$) isolates that formed part of the study describing the misclassification of the two bacteria (19) was

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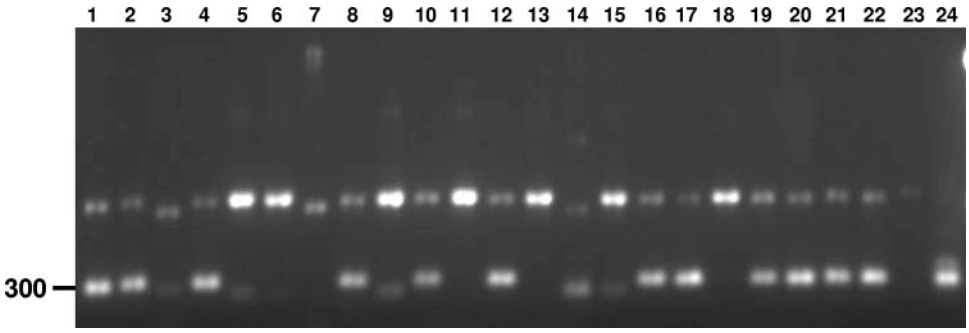


FIG. 1. Ethidium bromide-stained agarose gel showing electrophoretically separated PCR products generated with the oligonucleotide primers 3′- and 5′UNIVSOD with chromosomal DNA as the template. Lanes 1 to 20, *Haemophilus* isolates TIM1 to TIM20, respectively; lane 21, *H. haemolyticus* NCTC 10839; lane 22, *H. influenzae* biotype IV 16N; lane 23, *H. influenzae* Rd; lane 24, *N. meningitidis* MC58. The position of the predominant band at 300 bp, indicative of *sodC*, is indicated.

investigated. The *Haemophilus* isolates used in phase 1 of this study, which, for clarity, are given a short name in parentheses, were 1P26H (TIM1), 3P5H (TIM2), 3P16H1 (TIM3), 3P18H1 (TIM4), 5P1H (TIM5), 6P18H1 (TIM6), 7P49H1 (TIM7), 11P18H1 (TIM8), 14P41H1 (TIM9), 14P38H1 (TIM10), 24P19H1 (TIM11), 27P25H (TIM12), 48P38H1 (TIM13), 49P5H1 (TIM14), 55P13H1 (TIM15), 58P6H1 (TIM16), 65P13H1 (TIM17), 67P13H1 (TIM18), 70P28H1 (TIM19), and 73P18H1 (TIM20). This phase of the study was blind, with only one of the authors (T.F.M.) knowing the species designation. Strains used as positive controls were *H. haemolyticus* NCTC 10839, biotype IV cryptic genospecies *H. influenzae* strain 16N (16), *Neisseria meningitidis* MC58 (27), and *Actinobacillus pleuropneumoniae* 4074 ATCC 27088 (12, 13, 26); *H. influenzae* Rd (7, 11) was used as the negative control. *A. pleuropneumoniae* 4074 and *Haemophilus* isolates were grown in brain heart infusion (BHI) broth supplemented with 1 μg/ml NAD and 10 μg/ml hemin at 37°C on an orbital shaker (180 rpm) or on BHI agar plates supplemented with Levinthal's base at 37°C in 5% carbon dioxide (11). *N. meningitidis* was cultured as described previously (27). Chromosomal DNA was prepared from the plate-grown organisms (17), and standard methods were used for restriction endonuclease digestion and Southern blotting, with washing to 80% stringency (24). PCR amplification of genomic DNA with the degenerate primers 3′UNIVSOD and 5′UNIVSOD, designed to amplify a 300-bp conserved region of prokaryotic *sodC* genes, was performed as described previously (12), except that an annealing temperature of 44°C was used.

Use of the degenerate oligonucleotide primers and genomic DNA as the template resulted, as expected, in the PCR amplification of a number of fragments for each isolate (Fig. 1). However, for 10 isolates (TIM1, TIM2, TIM4, TIM8, TIM10, TIM12, TIM16, TIM17, TIM19, and TIM20) there was a band of 300 bp, indicative of the presence of a *sodC* gene. The strong 300-bp product was distinct from a fainter band of 280 bp present in isolates TIM3, TIM5, TIM6, TIM9, TIM14, and TIM15. The positive controls (*H. haemolyticus* NCTC 10839, *H. influenzae* 16N, and *N. meningitidis* MC58) amplified a corresponding 300-bp PCR product, while the negative control (*H. influenzae* Rd) did not. To confirm the presence or absence of *sodC*, as indicated by the PCR results, Southern blots were probed with a digoxigenin (DIG)-labeled 509-bp fragment from the *sodC* gene of *H. parainfluenzae* 1391 (12). The *H. parainfluenzae* internal *sodC* probe was labeled with a PCR DIG probe synthesis kit (Roche) by using oligonucleotides ATGATGAAAATGAAAACT and TGATCTGAGTGGTTATC with plasmid pJSK134 (12) as the DNA template. The *H. parainfluenzae sodC* probe hybridized to the EcoRI restriction fragment-digested genomic DNA of all the *Haemophilus* isolates when a 300-bp fragment had been amplified by PCR but not to the DNA of those isolates from which this band was absent (Fig. 2). The *sodC* probe hybridized to the positive controls (*H. haemolyticus* NCTC 10839 and *H. influenzae* biotype IV) but not the negative control (*H. influenzae* Rd). There were variations in the sizes of the EcoRI fragments that were hybridized by the *sodC* probe, indicative of genetic heterogeneity between such isolates.

Next we determined whether the CuZnSOD protein was produced by *Haemophilus* isolates. Whole-cell extracts were prepared as described previously (14, 15), except that the FastPrep

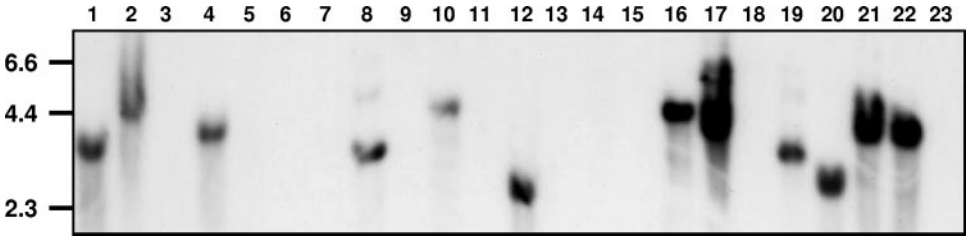


FIG. 2. Southern blot of EcoRI-digested chromosomal DNA restriction fragments hybridized to a full-length *H. parainfluenzae* 1391 *sodC* probe. Lanes 1 to 20, *Haemophilus* isolates TIM1 to TIM20, respectively; lane 21, *H. haemolyticus* NCTC 10839; lane 22, *H. influenzae* biotype IV 16N; lane 23, *H. influenzae* Rd. Size marker (in kilobases) are indicated on the left.

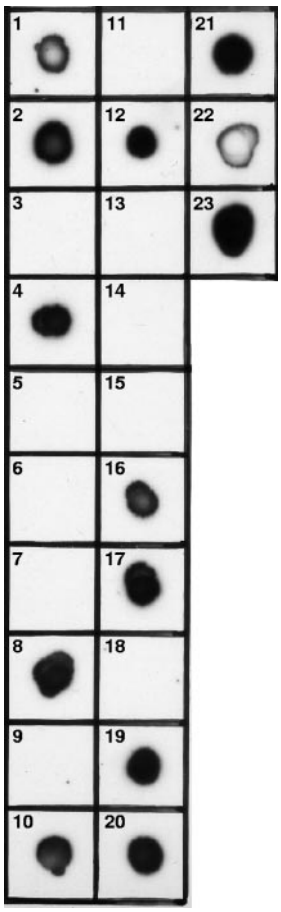


FIG. 3. Dot blot showing reactivities of whole-cell protein extracts with anti-CuZnSOD monoclonal antibody HD1. Grids 1 to 20, *Haemophilus* isolates TIM1 to TIM20, respectively; grid 21, *H. haemolyticus* NCTC 10839; grid 22, *H. influenzae* biotype IV 16N; grid 23, *A. pleuropneumoniae* 4074.

FP120 instrument (ThermoElectron) instead of sonication was used to disrupt the cells. The settings used were two runs at setting 6.0 for 40 s each, with each run separated by a 10-min cooling step at 4°C. Prior experiments had determined that this resulted in optimal cell breakage. The protein concentration was measured on an ND-1000 spectrophotometer (Nanodrop Technologies). Whole-cell extracts (12.5 μg) were spotted onto Hybond-C (GE Healthcare) membranes; and their reactivities with mouse monoclonal antibody HD1, which recognizes *Haemophilus*, *A. pleuropneumoniae*, and meningococcal CuZnSODs, were determined. The membranes were blocked for 30 min with 5% skim milk in phosphate-buffered saline (PBS) containing 0.3% Tween 20 (PBS-T). Primary antibody (HD1) was used at a 1:500 dilution of hybridoma supernatant (with incubation for 60 min), and secondary antibody (rabbit anti-mouse immunoglobulin–horseradish peroxidase conjugate; Dako) was used at a 1:10,000 dilution for 30 min. PBS-T was used for all washes except the final wash, which was done in PBS alone. The dot blots were developed with ECL-Plus (GE Healthcare), according to the manufacturer's instructions. Again, there was a perfect correlation between those isolates that possessed *sodC*, as determined by PCR or Southern blotting, and the presence of CuZnSOD, as indicated by the recognition by HD1 (Fig. 3).

Finally, we determined whether isolates possessed active CuZnSOD. This was achieved by separation of whole-cell extracts by isoelectric focusing (IEF) with pH 3 to 10 Ready gels (Bio-Rad) and SOD activity staining, coupled with differential inhibition, as described previously (15). The copper chelator diethyl dithiocarbamic acid (DEDC, 10 mM) was used as the inhibitor of CuZnSOD activity (3, 15). SOD gels demonstrating the presence of CuZnSOD activity in eight isolates (TIM1, TIM2, TIM4, TIM8, TIM10, TIM12, TIM16, and TIM17, also representative of TIM19 and TIM20), an example of a single strain (TIM18) representative of nine other strains (TIM3, TIM5 to TIM7, TIM9, TIM11, and TIM13 to TIM15), plus the positive control *A. pleuropneumoniae* 4074, are shown in Fig. 4. In the eight isolates containing CuZnSOD activity (TIM1, TIM2, TIM4, TIM8, TIM10, TIM12, TIM16, and TIM17; Fig. 4, lanes 1 to 8, respectively), two achromatic bands (arrowed) that disappear with DEDC are clearly visible. In contrast, the single band of SOD activity seen in TIM18 was unaffected by DEDC treatment. The positive control (*A. pleuropneumoniae* 4074) showed three bands of activity, two of which were DEDC inhibitable. Active CuZnSOD (DEDC inhibitable) was found only in those *Haemophilus* isolates that (i) contained a *sodC* gene, as indicated by the presence of the 300-bp PCR product by using 3′- and 5′UNIVSODs as primers or hybridization of the *H. parainfluenzae sodC* probe in Southern blotting experiments, and (ii) produced the CuZnSOD protein, as determined by reactivity with monoclonal antibody HD1. The presence of two bands with CuZnSOD activity in the IEF gels is probably due to the fact that *H. haemolyticus* SodC is dimeric.

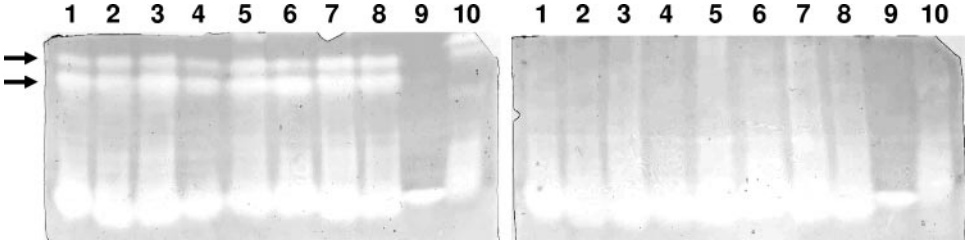


FIG. 4. Paired IEF gels stained to show SOD activity as an achromatic zone without selective CuZnSOD inactivation (left) and in the presence of the CuZnSOD inhibitor DEDC (right). The lanes contain whole-cell protein extracts, as follows: lanes 1, TIM1; lanes 2, TIM2; lanes 3, TIM4; lanes 4, TIM8; lanes 5, TIM10; lanes 6, TIM12; lanes 7, TIM16; lanes 8, TIM17; lanes 9, TIM18; lanes 10, *A. pleuropneumoniae* 4074. The arrows indicate the positions of the two achromatic bands in lanes 1 to 8 that disappear upon DEDC treatment.

HD1 does not recognize monomeric CuZnSODs from *Salmonella enterica* serovar Typhimurium or *Escherichia coli*. The monoclonal antibody does, however, recognize dimeric SODs, such as that from *A. pleuropneumoniae* 4074 (8), which also showed two bands on the IEF gel (Fig. 4). In Western blots, HD1 recognized a protein of approximately 50 kDa in all the *H. haemolyticus* isolates, i.e., TIM1, TIM2, TIM4, TIM8, TIM10, TIM12, TIM16, TIM17, TIM19, and TIM20, when there was reactivity with HD1 (data not shown). Given the presence of the 300-bp band upon PCR with 3′- and 5′UNIVSOD primers, designed to amplify a highly conserved C-terminal region of *sodC* genes, it is probable that the structure of the full-length protein of *H. haemolyticus* is similar to those established for other prokaryotic CuZnSODs. Typically, the molecular mass of the predicted encoded proteins for known monomeric CuZnSODs or a single subunit of dimeric SODs is 17 to 25 kDa (2, 15). Thus, the available evidence suggests that the *H. haemolyticus* CuZnSOD is dimeric, although this awaits definitive confirmation.

From these results with blinded isolates, isolates TIM1, TIM2, TIM4, TIM8, TIM10, TIM12, TIM16, TIM17, TIM19, and TIM20 were predicted to be *H. haemolyticus* and the remainder were predicted to be NT *H. influenzae*. When the code was broken, all of the predictions were found to be correct. To confirm the usefulness of *sodC*/CuZnSOD as a means of distinguishing between *H. haemolyticus* and NT *H. influenzae*, a further 20 strains were analyzed in a blinded manner. These were strains 1P67H1 (TIM21), 3P60H1 (TIM22), 5P54H1 (TIM23), 6P23H3 (TIM24), 7P24H1 (TIM25), 12P56H1 (TIM26), 13P13H5 (TIM27), 18P25H1 (TIM28), 19P49H1 (TIM29), 24P21H1 (TIM30), 26P1H1 (TIM31), 28P15H2 (TIM32), 31P13H1 (TIM33), 32P3H1 (TIM34), 34P4H6 (TIM35), 39P8H1 (TIM36), 43P2H1 (TIM37), 45P9H1 (TIM38), 47P68H1 (TIM39), and 50P5H1 (TIM40). In view of the 100% correlation between the results of PCR, Southern blotting, dot blotting, and CuZnSOD activity in the initial phase, only PCR and dot blotting with monoclonal antibody HD1 were performed. Isolates TIM21, TIM22, TIM24, TIM25, TIM27, TIM28, TIM30, TIM32, TIM34, and TIM36 were predicted to be *H. haemolyticus*, and the remainder were predicted to be NT *H. influenzae*. When the code was broken, 100% predictive accuracy was again achieved.

We conclude that the presence of a *sodC* gene or CuZnSOD protein can reliably differentiate between NT *H. influenzae* and *H. haemolyticus* strains isolated from the respiratory tract. A test based on the CuZnSOD activity of single bacterial colonies on agar plates or in broth based on a chromogenic reaction would be ideal; but this is challenging, as current methods do not distinguish between the three major bacterial forms of SOD (MnSOD, FeSOD, and CuZnSOD) and a reliable inhibitor for prokaryotic MnSODs is lacking. Nevertheless, while we await appropriate developments for simple rapid tests in the field of SOD assays, the procedures described herein can clearly distinguish *H. haemolyticus* from NT *H. influenzae*. A test that combines monoclonal antibodies HD1 (which recognized the CuZnSOD of *H. haemolyticus* but not that of NT *H. influenzae*) and 7F3 (which recognized the P6 protein of NT *H. influenzae* but not that of *H. haemolyticus*) (19) has some merit. Alternatively, a multiplex PCR based on *H. haemolyticus sodC* and a gene unique to NT *H. influenzae* might be developed. It should be noted that in a previous study representative strains of *H. parahaemolyticus* and *H. paraphrohaemolyticus* produced active CuZnSOD (15). Therefore, the presence of CuZnSOD cannot be used to distinguish *H. parahaemolyticus* or *H. paraphrohaemolyticus* from *H. haemolyticus*. We speculate that the CuZnSODs produced by both *H. parahaemolyticus* and *H. paraphrohaemolyticus* would be recognized by monoclonal antibody HD1, although this remains to be confirmed.

The finding that *H. haemolyticus*, a relatively newly recognized commensal of the lower respiratory tract, produces active CuZnSOD, while NT *H. influenzae* (which is associated with new exacerbations in COPD patients) does not suggests that SodC is not an important virulence factor, at least in the context of the lower respiratory tract. It is not known whether *H. haemolyticus* produces CuZnSOD in vivo, and its role in host interactive biology, if any, remains to be determined.

In summary we have described a marker (*sodC* and/or CuZnSOD) that differentiates *H. haemolyticus* from NT *H. influenzae*. While the methods used in the present study would be too time-consuming to become part of a routine laboratory protocol, they pave the way forward for the development of a simple rapid test.

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