Comparison of API 20E and *invA* PCR for Identification of *Salmonella enterica* Isolates from Swine Production Units

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API 20E and *invA* PCR were evaluated for the identification of *Salmonella enterica* isolates from swine farms. API 20E had the highest agreement with other tests at the 99.9% likelihood level. Both tests had 100% sensitivity and 96% specificity compared to 16S rRNA sequencing. Compared to serotyping, both tests had 96% sensitivity; specificity was 86% for API 20E and 79% for *invA* PCR.

The API 20E diagnostic, which detects 20 biochemical reactions, is a traditional method for the identification of *Salmonella enterica* and other *Enterobacteriaceae* (11). Previous studies of API 20E have reported both good (14, 16, 20) and inaccurate (1, 2, 9, 18) sample classifications. Genetic identification systems may improve *Salmonella* identification (8, 22). An alternative is the highly sensitive and specific *invA* PCR (5, 13). *invA* PCR accuracy for *S. enterica* detection needs to be confirmed for animal and environmental samples. The present study compared API 20E and *invA* PCR in the identification of *S. enterica*, using isolates from swine farms. Test results were validated by comparison with serotyping and 16S rRNA gene sequencing.

Three Illinois swine farms were each visited twice over a 3-month period (January to March 2003). The samples collected included feces from pigs and captured rodents, insects, pen floor contents, boot scrapings, feed, and water. Samples were cultured for *Salmonella* by using tetrathionate (Remel, Lenexa, KS) and then Rappaport-10 (Remel, Lenexa, KS) enrichment broths, plated on xylose-lysine-tergitol-4 (Remel, Lenexa, KS), and brilliant green (Remel, Lenexa, KS) selective and differential media. Suspect colonies were subcultured to tryptic soy agar plates (Difco, Detroit, MI).

For each isolate, an API 20E strip (bioMérieux, Inc., Hazelwood, MO) was inoculated and incubated according to the manufacturer's instructions. The likelihood of *S. enterica* was calculated, using the manufacturer's coding system, based on reactions to reagents in the 20 compartments.

Template DNA for amplification was prepared by adding a tryptic soy agar plate colony sample to 100 μ l sterile Millipore water and boiling for 5 min. An oligonucleotide primer set, producing a 244-bp amplified fragment, was selected from a published *S. enterica invA* gene sequence (5). For all assays, 2 μ l of template was added to a 23- μ l PCR mixture, containing 0.4 μ M of each primer (Integrated DNA Technologies, Inc., Coralville, IA), 250 μ M deoxynucleoside triphosphates (TaKaRa Bio Inc., Otsu, Shiga, Japan), 1 $\times$ PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl₂), and 0.5

units of TaKaRa recombinant *Taq* (TaKaRa Bio Inc., Otsu, Shiga, Japan). Amplification conditions were as described previously (5).

A 16S rRNA primer (*rrs*) set (Integrated DNA Technologies, Inc.), amplifying a 320-bp fragment common to all bacteria (21), served as the DNA template quality control. A 0.1 μ M concentration of each *rrs* primer was added to each PCR. Amplicons were separated by gel electrophoresis and photographed under UV illumination.

For comparison, two additional primer sets were used for the same template preparations, following published protocols. The *fimY* primer set (Integrated DNA Technologies, Inc.) amplifies a 526-bp fragment unique to *Salmonella* (23). Another *invA* primer set (Integrated DNA Technologies, Inc.), targeting a different region, produces a 284-bp fragment (17).

All isolates classified as *S. enterica* by API 20E with likelihood values from 62.5% to 99.9% were serotyped for confirmation (Salmonella Reference Center, University of Pennsylvania New Bolton Center, Kennett Square, PA). These isolates were also sequenced, using primers amplifying a 505-bp fragment from the 16S rRNA gene (12). Electrophoresis conditions were the same as those for *invA* PCR. The amplicons were gel extracted using the QIAquick gel extraction kit (QIAGEN Sciences, Maryland) and sequenced (Roy J. Carver Biotechnology Center, University of Illinois) using forward and reverse primers. Sequences were submitted to a BLASTn sequence similarity search (<http://www.ncbi.nlm.nih.gov>) (3) and classified as *S. enterica* only if there was 100% agreement with sequences classified as *Salmonella* in the database.

The validities of *invA* PCR and API 20E isolate classifications were evaluated by comparison to serotyping and 16S rRNA sequencing as diagnostic standards. Six API 20E likelihood levels (99.9%, 95%, 90%, 85%, 75%, and 70%) were examined. Agreement among tests (kappa coefficient) was calculated (6), with statistical significance ($\alpha = 0.01$) assessed using the Fisher exact test (7).

Of 196 suspected *S. enterica* isolates, API 20E identified 174 as *S. enterica*, with likelihood values from 62.5% to 99.9%. The most common serotypes were Derby (29%), Typhimurium (23%), London (20%), and Infantis (10%); 34 isolates (20%) were classified as "rough" or "untypeable." Both *invA* PCR assays and the *fimY* PCR produced identical classification of suspect isolates, identifying 125 as *S. enterica*.

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TABLE 1. Sensitivity and specificity of API 20E at 99.9% likelihood and *invA* PCR compared to serotyping and 16S rRNA sequencing as diagnostic standards

Test and result	No. of isolates tested by:					
	16S rRNA sequencing			Serotyping		
	Positive	Negative	Total	Positive	Negative	Total
API 20E						
Positive	123	2	125	121	2	123
Negative	0	49	49	5	12	17
Total	123 (100) ^a	51 (96) ^b	174	126 (96) ^a	14 (88) ^b	140
<i>invA</i> PCR						
Positive	123	2	125	121	3	124
Negative	0	49	49	5	11	16
Total	123 (100) ^a	51 (96) ^b	174	126 (96) ^a	14 (79) ^b	140

^a Value in parentheses is a percentage indicating sensitivity.

^b Value in parentheses is a percentage indicating specificity.

There were 125 isolates positive at the API 20E 99.9% likelihood level (API-99); 124 were also positive by *invA* PCR, 123 by 16S rRNA sequencing, and 121 by serotyping. None of the 49 isolates positive for API 20E at lower likelihood levels had *Salmonella* 16S rRNA sequences; however, five (all at 79.6% API 20E level) were serotyped as *S. enterica*. The sensitivities of both API-99 and *invA* PCR were 100% compared to that of 16S rRNA sequencing; the specificity was 96% for both tests (Table 1). Compared to that of serotyping, the sensitivity of both tests was 96%, whereas specificity was 86% for API-99 and 79% for *invA* PCR.

Agreement between API 20E and 16S rRNA sequencing decreased with a decrease in API likelihood level ($\kappa = 0.97, 0.91, 0.77, 0.75$, and 0.3 at API levels of 99%, 95%, 90%, 85%, and 75%, respectively). There was near-perfect agreement between API-99 and *invA* PCR ($\kappa = 0.99$); one isolate classified as *S. enterica* by API-99 was negative with *invA* PCR. There was less than perfect agreement between 16S rRNA sequencing and serotyping ($\kappa = 0.87$); five isolates classified as *S. enterica* by serotyping (three Derby, one Seftemberg, and one Typhimurium) had 99 to 100% sequence similarity with the 16S rRNA gene of both *Citrobacter freundii*. All of these isolates were negative by *invA* PCR and classified by API 20E as *Salmonella* at the low discrimination level (79.6% likelihood).

The near-perfect sensitivity and specificity of both API-99 and *invA* PCR compared to those of 16S rRNA sequencing indicate that both tests can provide accurate diagnoses for isolates obtained through culture on selective media for *S. enterica*. Previous studies also have accepted only high API 20E likelihood levels as accurate (>90% correct) in the classification of suspected *Salmonella* isolates (4, 10). The present study, using 16S rRNA sequencing as a diagnostic standard, indicates that caution should be exercised in classifying as *S. enterica* any result from API 20E for which the likelihood of *Salmonella* is less than 99.9%.

The reliability of the results presented above was enhanced by complete agreement between the two *invA* primer sets used as well as with the *fimY* PCR. These findings affirm previous studies finding *invA* PCR to be a reliable, accurate tool in the detection of *S. enterica* (5, 13, 15, 19).

With 20% of isolates serotyped as “rough” or “untypeable,”

this suggests a limitation in the precision of *Salmonella* serology. In a previous study, 10% of isolates from a veterinary diagnostic laboratory were “rough,” with classification of these isolates as *Salmonella* possible through *invA* PCR (8). With five isolates serotyped as *Salmonella*, yet not classified by 16S rRNA sequencing as *Salmonella*, there is also a risk of false positives using serotyping.

Validation of both *invA* PCR and API 20E (at the 99.9% likelihood level) as accurate diagnostic tests suggests that either can be used with similar results. The selection of *invA* PCR versus API 20E depends in part upon cost. API 20E strips are relatively expensive, currently about \$6 per sample, compared to less than \$2 current cost in laboratory materials per *invA* PCR test. There are also reduced labor costs using *invA* PCR. However, PCR requires an initial investment in equipment (e.g., thermocyclers and electrophoretic gel apparatus). Another cost saving for API 20E is that each sample can be tested individually; with PCR, cost efficiency is realized only when samples are tested in quantities sufficient to populate a gel for electrophoresis. Thus, in small laboratories with limited equipment and low sample numbers, API 20E may still be economical. However, in larger well-equipped laboratories with higher sample numbers, *invA* PCR becomes more economical. In any case, *invA* PCR has the additional advantage of obtaining results within hours of isolation of *Salmonella* suspects on selective media and may even be used to detect *Salmonella* in enrichment broth. The interpretation of results is straightforward, avoiding problems of subjective evaluation of API 20E biochemical reactions. However, *invA* PCR is able to identify only *S. enterica* and not other *Enterobacteriaceae* as does API 20E, thus limiting its identification to one specific pathogen.

In summary, both *invA* PCR and API 20E (at the 99.9% likelihood level) were demonstrated to be accurate methods for *S. enterica* identification. The results of this study of samples from animal production systems are in general agreement with those of previous studies of samples collected for diagnosis in both veterinary and human medicine, thus indicating wide applicability of both diagnostic tools for *Salmonella* identification.

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