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Research Note—

Characterizing Avian *Escherichia coli* Isolates with Multiplex Polymerase Chain Reaction

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SUMMARY. Colibacillosis caused by *Escherichia coli* infections account for significant morbidity and mortality in the poultry industry. Yet, despite the importance of colibacillosis, much about the virulence mechanisms employed by avian *E. coli* remains unknown. In recent years several genes have been linked to avian *E. coli* virulence, many of which reside on a large transmissible plasmid. In the present study, a multiplex polymerase chain reaction (PCR) protocol to detect the presence of four of these genes is described. Such a protocol may supplement current diagnostic schemes and provide a rapid means of characterizing the *E. coli* causing disease in poultry. The targets of this procedure included *iss*, the increased serum survival gene; *tsb*, the temperature sensitive hemagglutinin gene; *cvi*, the ColV immunity gene; and *iucC*, a gene of the aerobactin operon. Organisms, known for their possession or lack of these genes, were used as a source of the template DNA to develop the multiplex PCR protocol. Identity of the amplicons was confirmed by size, DNA:DNA hybridization with specific gene probes, and DNA sequencing. When the multiplex PCR protocol was used to characterize 10 *E. coli* isolates incriminated in avian colibacillosis and 10 from the feces of apparently healthy birds, nine of the isolates from apparently healthy birds contained no more than one gene, while the 10th contained all four. Also, eight of the isolates incriminated in colibacillosis contained three or more genes, while the remaining two contained two of the target genes. Interestingly, the isolates of sick birds containing only two of the targeted genes killed the least number of embryos, and the isolate of healthy birds that contained all the genes killed the most embryos among this group. These genes were not found among the non-*E. coli* isolates tested, demonstrating the procedure's specificity for *E. coli*. Overall, these results suggest that this protocol might be useful in characterization and study of avian *E. coli*.

RESUMEN. *Nota de Investigación*—Caracterización de cepas aviarias de *Escherichia coli* mediante la técnica de reacción en cadena por la polimerasa de amplificación múltiple.

La colibacilosis debida a infecciones por *Escherichia coli* es causa de niveles significativos de mortalidad y morbilidad en la industria avícola. A pesar de la importancia de la enfermedad, se desconocen muchos de los mecanismos de virulencia de las cepas aviarias de *E. coli*. En años recientes se han descubierto varios genes implicados en mecanismos de virulencia de la bacteria, muchos de los cuales residen en un plásmido transmisible de gran tamaño. En este estudio se utilizó un protocolo basado en la técnica de reacción en cadena por la polimerasa de amplificación múltiple (multiplex PCR por sus siglas en Inglés), con el fin de detectar la

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presencia de cuatro de estos genes en aislados aviares. Este protocolo podría servir como complemento de los esquemas diagnósticos utilizados actualmente y podría ser una forma rápida de caracterizar las cepas de *E. coli* causantes de enfermedad en pollos. Los genes elegidos para el estudio fueron el *iss*, gen de supervivencia aumentada en suero; *tsh* gen de la hemaglutinina sensible a temperatura; *cvi*, gen de inmunidad a CoIV; e *iucC*, un gen del operón del aerobactin. Se utilizó ADN de organismos positivos y negativos para la presencia de estos genes con el fin de desarrollar el protocolo de la reacción. La identidad de los productos amplificados se determinó mediante el tamaño de los mismos, hibridación de ADN:ADN con sondas específicas para cada gen y mediante secuenciación de las moléculas de ADN. Cuando el protocolo de reacción en cadena por la polimerasa de amplificación múltiple se utilizó para caracterizar 10 cepas de *E. coli* aisladas a partir de aves que presentaba colibacilosis y 10 cepas obtenidas de muestras de heces de aves aparentemente sanas, 9 de las cepas obtenidas de muestras de heces en las aves sanas presentaron solo uno de los genes descritos, mientras que la cepa restante presentó los cuatro genes. Ocho de las cepas aisladas a partir de las aves enfermas presentaron tres o más de los genes estudiados, mientras que las dos cepas restantes presentaron solo dos de los genes de virulencia. En el estudio de letalidad embrionaria las cepas obtenidas de aves enfermas que solo presentaron dos de los genes de virulencia fueron las que causaron letalidad en el menor número de embriones, mientras que las cepas que presentaron los cuatro genes de virulencia aisladas a partir de las aves sanas causaron letalidad en el mayor número de embriones entre los grupos estudiados. Los genes de virulencia descritos no fueron encontrados en las otras especies de bacterias utilizadas como control negativo, lo que demuestra la especificidad del procedimiento para los aislados de *E. coli*. Estos resultados sugieren que este protocolo puede ser útil en la caracterización y estudio de las cepas aviares de *E. coli*.

Key words: *Escherichia coli*, avian colibacilosis, multiplex PCR, virulence, *iss*, *tsh*, *iucC*, *cvi*

Abbreviations: bp = base pair; CFU = colony forming unit; CoIV = colicin V; DNA = deoxyribonucleic acid; ddH₂O = double distilled water; EDTA = ethylenediaminetetraacetic acid; LB = Luria-Bertani; NDVDL = North Dakota Veterinary Diagnostic Lab; PBS = phosphate-buffered saline; PCR = polymerase chain reaction

Colibacilosis poses a major economic problem to the poultry industry. In spite of the costly nature of this disease, *Escherichia coli* has poorly described virulence mechanisms. In this study, a multiplex polymerase chain reaction (PCR) protocol was developed for characterization of the *E. coli* capable of causing avian colibacilosis in an effort to facilitate study of this pathogen. This PCR protocol targets four genes: *iss*, the increased serum survival gene (1); *tsh*, which encodes a temperature sensitive hemagglutinin (9); *iucC*, a gene encoding a protein involved in aerobactin production (2); and *cvi*, the colicin V immunity gene (4). These genes were chosen because of their association with avian *E. coli* virulence and their possible linkage to the same plasmid (3,7). Studies have shown that *iss* and *tsh* genes are found in a significantly higher percentage of *E. coli* isolates isolated from birds with colibacilosis than from apparently healthy birds (9,12). Earlier studies have also shown that *iss* and *tsh* genes reside on transmissible R plasmids encoding aerobactin and colicin V (3,7,16). Because of the possible utility of these genes as predictors and descriptors of

avian *E. coli* virulence, the multiplex PCR protocol described here targets *iss*, *tsh*, *iucC*, and *cvi*.

MATERIALS AND METHODS

Test organisms. Twenty avian *E. coli* isolates were used in this study. Ten originated from birds with colibacilosis (VA-VJ), and 10 originated from the feces of apparently healthy birds (NA-NJ) (10). The identity of *E. coli* isolates was confirmed using API20E strips (bioMérieux, Vitek, Inc., Hazelwood, MO), and the isolates were serotyped by the Pennsylvania State University *Escherichia coli* Reference Center (University Park, PA). The following non-*E. coli* strains were obtained from the North Dakota Veterinary Diagnostic Laboratory and were used to evaluate the specificity of the PCR protocol for *E. coli*: *Salmonella newport*, *Micrococcus luteus*, *Pseudomonas fluorescens*, *Rhodococcus equi*, *Enterobacter cloacae*, *Staphylococcus epidermidis*, *Klebsiella oxytoca*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Streptococcus faecalis*, *Staphylococcus aureus*, *Enterobacter aerogenes*, *Campylobacter jejuni*, and *Salmonella typhimurium*.

Media. All isolates were stored in Luria-Bertani

Table 1. Primers used in amplification of the *iss*, *tsh*, *iucC*, and *cvi* genes.

Name	Sequences (5' to 3')	Temp. (C)	Amplicon size (bp)	Ref.
<i>iss</i> ^A	Upper GTGGCGAAAACCTAGTAAACAGC	52.1	760	6
	Lower CGCCTCGGGGTGGATAA	53.9		
<i>tsh</i> ^B	Upper GGGAAATGACCTGAATGCTGG	67.6	420	9
	Lower CCGCTCATCAGTCAGTACCAC	64.6		
<i>iucC</i> ^C	Upper CGCCGTGGCTGGGGTAAAG	71.8	541	This study
	Lower CAGCCGGTTCACCAAGTATCACTG	70.4		
<i>cvi</i> ^D	Upper GGGCCTCCTACCCTTCACTCTTG	69.8	366	This study
	Lower ACGCCCTGAAGCACCACCAGAA	73.9		

^AGenBank accession number AF042279.

^BGenBank accession number AF218073.

^CGenBank accession number X76100.

^DGenBank accession number AF062858.

broth (Difco Laboratories, Detroit, MI) with 20% glycerol at -80°C prior to use (14). In preparation for amplification, isolates were grown on either MacConkey or Nutrient agar (Difco) overnight at 37°C .

Multiplex amplification protocol. Test and control organisms were examined for *iss*, *tsh*, *iucC*, and *cvi* genes using the following protocol. Primer sequences are listed in Table 1. Appropriate primer sequences to be used in PCR amplification of *tsh* and *iss* were obtained from earlier studies (6,9), and those for *cvi* and *iucC* were selected using Lasergene software (DNASTar, Inc., Madison, WI) on the basis of published sequences for these genes (5,8). Primers were obtained from Sigma Genosys, Inc. (The Woodlands, TX). Template DNA was obtained by placing a single colony of *E. coli* into 40 μl of a lysing buffer (10 mM Tris-Cl, pH 7.5, 1 mM ethylenediaminetetraacetic acid [EDTA], with 50 $\mu\text{g}/\text{ml}$ proteinase K added the day of use). This mixture was heated in a thermocycler at 55°C for 10 min, followed by 10 min at 80°C . Then, 80 μl of double distilled water (ddH_2O) was added, and the mixture was centrifuged for 30 sec at $10,000 \times g$. Five microliters of the supernatant fluid were used as the DNA template. Next, 45 μl of a master mix containing 28.5 μl H_2O ; 5 μl of $10 \times$ PCR Buffer (Invitrogen, Inc., Gaithersburg, MD); 4 μl of 1.25 mM deoxynucleotide triphosphate mix (Invitrogen, Inc.); 0.3 μl of *Taq* polymerase (5 U/ μl) (Invitrogen, Inc.); 0.3 μl of *iss* upper and lower primers; 0.1 μl of *tsh*, *iucC*, and *cvi* upper and lower primers (before addition to master mix all primers were at 0.1 mM); and 6 μl of 50 mM MgCl_2 was placed in a tube in a thermocycler (Perkin Elmer 2400; Perkin Elmer, Boston, MA) and allowed to heat to 90°C . Five microliters of template DNA was then added to the master mix in the thermocycler. Amplification was performed according to the following parameters: 5 min at 95°C , nine cycles of 60 sec at 95°C , 30 sec at 55°C , and 60 sec at 72°C ; 28 cycles of 30 sec at 94°C , 30 sec at 55°C , and 30 sec at 72°C ; 7

min at 72°C ; and a final soak at 4°C . The products were then separated in a 1.5% agarose gel, and a 1-kb ladder (Invitrogen, Inc.) was used as a size reference. For establishment of the protocol, *E. coli* strain V-B (10), thought to contain the target genes, was used to generate amplicons, which were identified by size, probing, and sequencing. The amplicons generated using the remaining *E. coli* isolates (VA–VJ, NA–NJ), as a source of template DNA, were identified by size alone. Isolates containing amplicons of the size predicted for each gene were considered to contain that gene (760 base pairs [bp] for *iss*, 420 bp for *tsh*, 541 bp for *iucC*, and 366 for *cvi*) (Table 2). The multiplex procedure was repeated three times for the 20 isolates.

Probe construction. DNA to be labeled for use as probes for the *iss*, *tsh*, *cvi*, and *iucC* sequences was obtained by DNA amplification techniques from *E. coli* strain V-B (previously described as V-2 in reference 11). Amplification products used to make probes were separated by horizontal gel electrophoresis in 1% low-melt agarose. Appropriate amplicons were identified by size, excised from agarose, and purified with GENE-CLEAN II (Bio101, La Jolla, CA). All DNA used in probe construction was labeled with a nonradioactive, random-primed DNA labeling kit (genius I labeling and detection kit; Roche, Indianapolis, IN).

Hybridization studies. Presumptive identification of amplicons generated in the multiplex PCR protocol was made by size and through the use of DNA:DNA hybridization studies. The hybridization studies were performed by blotting DNA in gels to charge-modified nylon membranes (Qiabran Nylon Plus, Qiagen Inc., Santa Clarita, CA) by the method of Southern (15). Membranes were prehybridized in aqueous solution (prehybridization solution: 5 Prime—3 Prime, Inc., Boulder, CO) for 4 hr at 68°C and hybridized with the individual denatured probes at 68°C for 12 hr according to the principles of Sambrook *et al.* (13). The filters were given two 1-hr

Table 2. Multiplex PCR, serotype, and lethality to chick embryos.

Isolate	O Group ^A	Embryo lethality				
		%	<i>iss</i>	<i>tsh</i>	<i>iucC</i>	<i>cvi</i>
N-A	—	9	+	+	+	+
N-B	—	6	—	—	—	—
N-C	69	1	—	—	—	—
N-D	32	0	—	—	—	—
N-E	—	3	—	—	—	+
N-F	—	1	—	—	—	+
N-G	108	3	—	—	—	—
N-H	M	3	—	—	—	—
N-I	—	3	—	—	—	+
N-J	27	5	—	—	—	—
V-A	—	59	+	+	+	+
V-B	2	15	+	+	+	+
V-C	—	47	+	+	+	+
V-D	—	6	+	—	—	+
V-E	78	51	+	+	+	+
V-F	11	9	+	—	+	—
V-G	2	59	+	+	+	+
V-H	—	50	+	—	+	+
V-I	78W	56	+	+	+	+
V-J	—	11	+	+	+	+

^A(—) in the O group column indicates a nontypeable strain.

washes in 0.1 × sodium chloride/sodium citrate with 0.1% sodium dodecyl sulfate (Amresco, Solon, OH). Hybridized probes were detected by the protocol in the genius I kit.

Sequencing. Identity of the multiplex PCR amplicons was confirmed by DNA sequencing using the IRDye 800 terminator sequencing protocol provided by LI-COR.

Sensitivity and specificity of the multiplex PCR protocol. The sensitivity of the multiplex PCR protocol was determined in the following manner. An *E. coli* isolate that contained all four genes (V-B) (11) was incubated overnight in Luria–Bertani (LB) broth, and the number of colony-forming-units per milliliter was determined using viable plate counts performed in triplicate. A 1:2 dilution series was then made from this broth. Fifty microliters of each dilution were placed in a 200 µl-PCR microfuge tube. These samples were then centrifuged at 10,000 × *g* for 5 min. The supernatant fluid from each tube was discarded, and the pellet used to generate the template DNA to be used in the multiplex PCR protocol. Amplification was undertaken as described previously, and the success of the amplification was evaluated in relation to the number of bacterial cells serving as a source of template DNA. The specificity of the assay for *E. coli* was determined by subjecting a number of bacterial strains, other than *E. coli*, to the multiplex PCR protocol.

Embryo lethality assay. Isolates were assessed previously for lethality in chicken embryos by inoculation of overnight washed bacterial cultures into the allantoic cavity of 12-day-old embryonated specific-pathogen-free eggs (17). One hundred embryos were used per organism, and phosphate-buffered saline (PBS)-inoculated and -uninoculated embryos were used as controls. Embryo deaths were recorded for 4 days.

RESULTS AND DISCUSSION

It has been reported elsewhere that *iss* and *tsh* are found in a much larger percentage of disease-associated *E. coli* than in isolates obtained from apparently healthy birds and that these genes may be found on a large aerobactin-encoding colicin V-producing R plasmid (7,9,12). Targeting *iss*, *tsh*, and genes of the aerobactin and colicin V operons, a multiplex PCR assay was developed that might have utility in characterizing and identifying outbreak strains of avian *E. coli*. The genes targeted in this assay have been localized to a large transmissible R plasmid described by Johnson *et al.* (7). The multiplex PCR primers were designed to facilitate both the ready differentiation and concomitant amplification of the PCR products. Although the *cvi* and *tsh* amplicons appear relatively close in size, they are easily resolved when compared to a standard 1-kb ladder (Fig. 1). The *tsh* amplicon lies above the 396-bp band, and the *cvi* amplicon lies below the 396-bp band.

For development of this test, identity of V-B's amplicons was verified in several ways. The identity of these amplicons was confirmed by size (Fig. 1), homology to specific probes (Fig. 2), and sequencing (data not shown). Sequencing results reflected the published sequences of these genes. Also, the specificity of the test for *E. coli* was determined. None of the non-*E. coli* strains, when used as a source of template DNA, produced amplicons of appropriate sizes using the multiplex PCR protocol described. No attempt was made to test the assay's specificity to virulent *E. coli* from other hosts since some of these genes have been previously found in nonavian *E. coli* (16), suggesting that these genes could be expected in *E. coli* from a variety of hosts. The sensitivity of this assay was also determined by relating the number of colony-forming units (CFUs) used to generate template with the ability to detect all four amplicons. The fewest bacterial cells that provided for visible amplification of all genes was 2500 CFUs per 50 µl reaction; however,

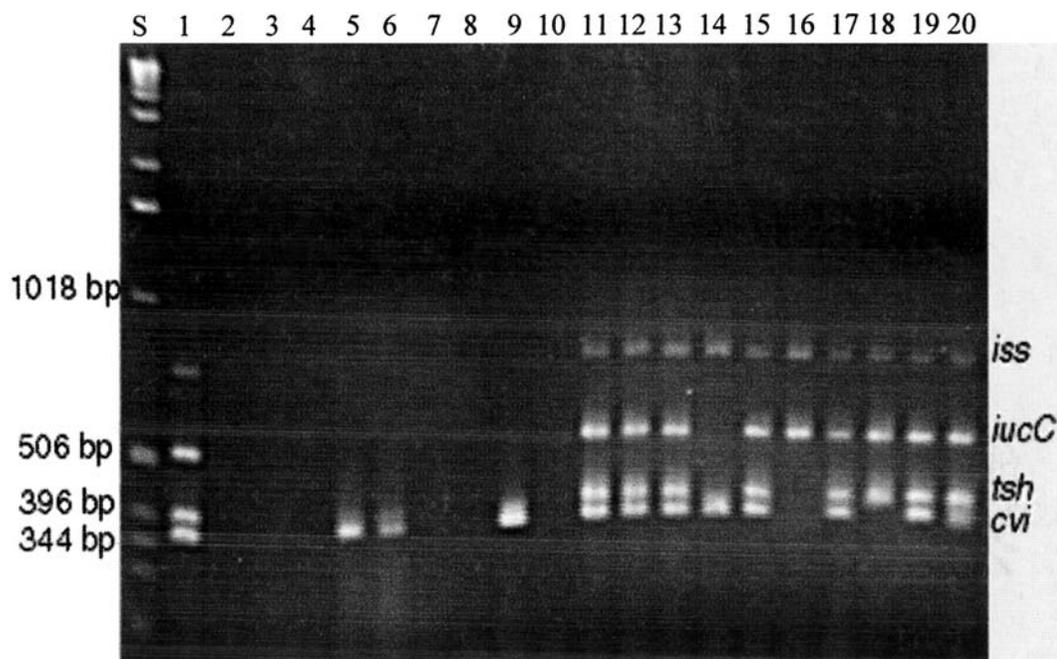


Fig. 1. Agarose gel of amplicons generated in the multiplex PCR protocol using the test *E. coli* isolates as a source of template DNA. Lane S contains a 1-kb ladder. Lanes 1–10 contain isolates NA–NJ from healthy birds, and lanes 11–20 contain isolates VA–VJ from sick birds.

other methods of DNA template preparation may be able to increase the sensitivity. Additionally, the multiplex procedure was repeated three times using a single DNA preparation from all 20 test *E. coli* strains and for the non-*E. coli* strains. In no case did the results differ among runs.

This multiplex protocol was developed to facilitate rapid characterization of the *E. coli* capable of causing disease in poultry. Twenty well-described avian *E. coli* isolates (Table 2) were characterized using this multiplex procedure in an effort to assess its utility for analysis of such strains. All isolates had previously been tested for virulence in chick embryos before being assayed with the multiplex PCR protocol (Table 2). Eight of ten virulent *E. coli* isolates contained at least three out of the four target genes, while the remaining two isolates from sick birds contained two genes. Interestingly, these two isolates, containing only two genes, were found to kill fewer chick embryos than the other isolates from sick birds (Table 2). Of the 10 isolates obtained from apparently healthy birds, 9 contained one gene or fewer (Table 2). Of interest is the 10th isolate, which contained all four genes. This isolate killed more embryos than the other isolates from healthy birds and killed as many or more embryos than two of the isolates of sick birds. This

result suggests that although this isolate was obtained from the feces of a healthy bird, it may be able to cause disease if introduced into a vulnerable host. Overall, it appears in this 20-isolate sample that the prevalence of these genes is higher in isolates from sick birds and that a relationship between the number of genes present and virulence for chick embryos may exist (Table 2). It will be interesting to determine whether this relationship can be verified in a larger sample of isolates.

This protocol may also prove to be a cost-effective means of characterizing avian *E. coli* isolates. When performing this multiplex PCR assay on 92 samples and four controls, about 2 hr of actual benchwork is required to obtain template DNA from the isolates, prepare the master mix, load master mix/DNA template mixture in the thermocycler, add loading buffer to samples, load samples into agarose gels, electrophorese samples, stain gels, and take pictures of the gels. Additionally, about 5 hr are required for the completion of cycling protocol, electrophoresis, and staining of amplicons. Altogether, the multiplex PCR protocol performed as described here would require 7 hr and would generate data for four genes within each isolate. A similar amount of time would be required to generate data on this number of

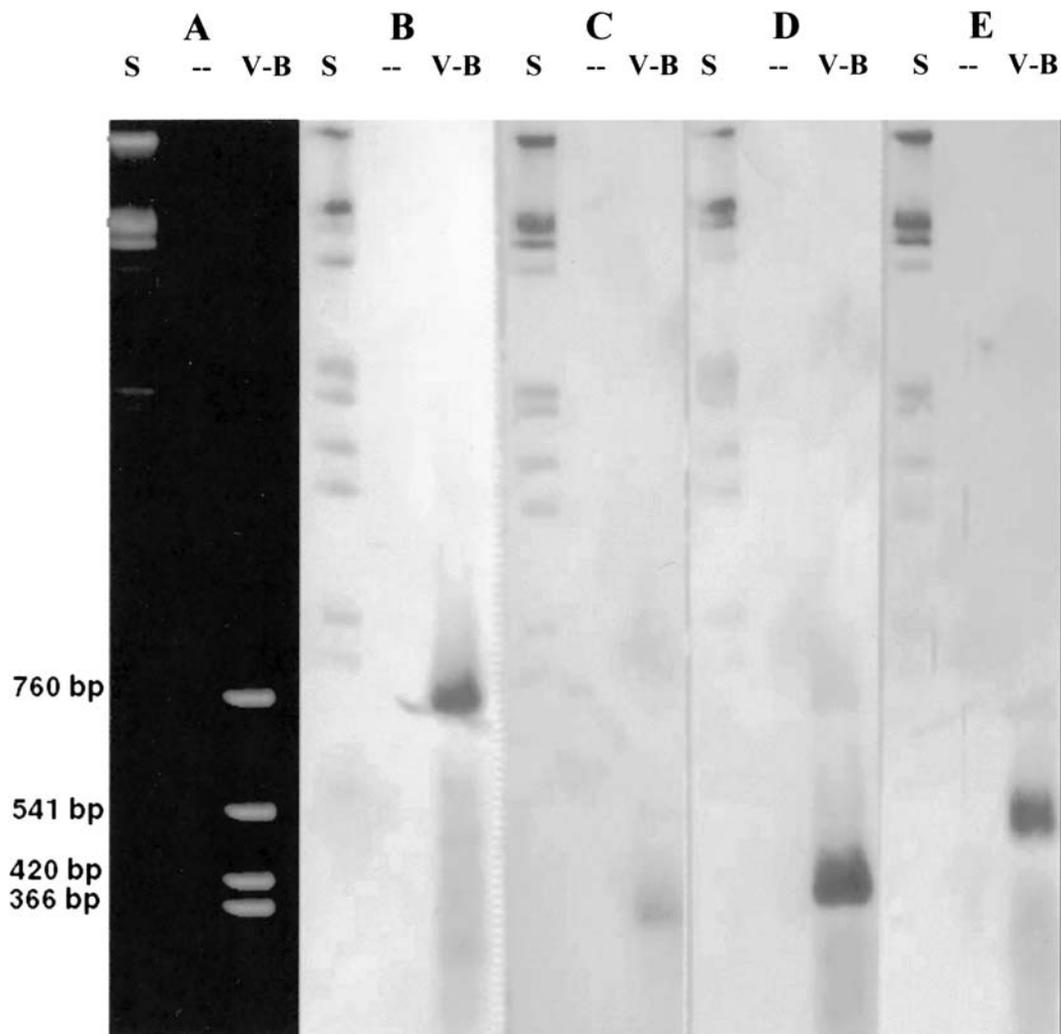


Fig. 2. Southern hybridization of multiplex PCR product containing all four genes. S = 1-kb ladder; -- = DNA from *E. coli* DH5 α (7,13), known to lack all the genes targeted in the multiplex procedure; V-B = virulent test *E. coli* (10), known to contain all the genes targeted in the multiplex procedure; A = gel containing amplicons that were blotted to nylon; and B, C, D, E = blots of gels identical to gel A and hybridized with various gene probes; blot B = Probed with labeled *iss*; blot C = probed with labeled *cvi*; blot D = probed with labeled *tsb*; blot E = probed with labeled *iucC*.

isolates for a single gene using standard procedures. Therefore, we estimate that assaying isolates with this multiplex procedure, rather than doing each gene individually, would save 6 hr of actual technician time and 15 hr of wait time per 96 samples. The multiplex PCR protocol uses 1.5 units of *Taq* polymerase per sample, while single target assays in our lab use about 1.25 units of *Taq*. Therefore, when targeting four genes, the multiplex PCR protocol uses 1.5 units of *Taq* per sample,

whereas the same results using single target assays would require 5 units of *Taq* per sample. Therefore, 3.5 units of *Taq* polymerase can be saved per sample, resulting in a substantial monetary savings over time. This multiplex PCR protocol might also be modified for real time PCR, resulting in a considerable time savings over the multiplex procedure described here, since the need for electrophoresis of samples in gels and the staining of the gels is eliminated.

With few exceptions among the isolates studied here, it appears that the more virulent an isolate is for embryos, the more of the targeted genes an avian *E. coli* contains. Further research may refine the predictive nature of this test. Therefore, we feel this protocol might prove useful as a tool to characterize outbreak strains of avian *E. coli*. This multiplex PCR assay may have value for diagnosticians and researchers interested in a cost-effective and time-efficient means of studying avian *E. coli*.

REFERENCES

1. Binns, M. M., J. Mayden, and R. P. Levine. Further characterization of complement resistance conferred on *Escherichia coli* by the plasmid genes *traT* of R100 and *iss* of ColV, I-K94. *Infect. Immun.* 35:654–659. 1982.
2. DeLorenzo, V., and J. B. Neilands. Characterization of *iucA* and *iucC* genes of the aerobactin system of plasmid ColV-K30 in *Escherichia coli*. *J. Bacteriol.* 167:350–355. 1986.
3. Dozois, C. M., M. Dho-Moulin, A. Bree, J. M. Fairbrother, C. Desautels, and R. Curtiss III. Relationship between the Tsh autotransporter and pathogenicity of avian *Escherichia coli* and localization and analysis of the *tsh* genetic region. *Infect. Immun.* 68:4145–4154. 2000.
4. Gilson, L., H. K. Mahanty, and R. Kolter. Four plasmid genes are required for colicin V synthesis, export, and immunity. *J. Bacteriol.* 169:2466–2470. 1987.
5. Gilson, L., H. K. Mahanty, and R. Kolter. Genetic analysis of an MDR-like export system: the secretion of colicin V. *Embo. J.* 9:3875–3894. 1990.
6. Horne, S. M., S. J. Pfaff-McDonough, C. W. Giddings, and L. K. Nolan. Cloning and sequencing of the *iss* gene from a virulent avian *Escherichia coli*. *Avian Dis.* 44:179–184. 2000.
7. Johnson, T. J., C. W. Giddings, S. M. Horne, P. S. Gibbs, R. E. Wooley, J. Skyberg, P. Olah, R. Kercher, J. S. Sherwood, S. L. Foley, and L. K. Nolan. Location of increased serum survival gene and selected virulence traits on a conjugative R plasmid in an avian *Escherichia coli* isolate. *Avian Dis.* 46:342–352. 2002.
8. Martinez, J. L., M. Herrero, and V. de Lorenzo. The organization of intercistronic regions of the aerobactin operon of pColV-K30 may account for the differential expression of the *iucABCD iutA* genes. *J. Mol. Biol.* 238:288–293. 1994.
9. Maurer, J. J., T. P. Brown, W. L. Steffens, and S. G. Thayer. The occurrence of ambient temperature-regulated adhesins, curli, and the temperature-sensitive hemagglutinin Tsh among avian *Escherichia coli*. *Avian Dis.* 42:106–118. 1998.
10. Nolan, L. K., C. W. Giddings, S. M. Horne, C. Doetkott, P. S. Gibbs, R. E. Wooley, and S. L. Foley. Complement resistance, as determined by viable count and flow cytometric methods, and its association with the presence of *iss* and the virulence of avian *Escherichia coli*. *Avian Dis.* 46:386–392. 2002.
11. Nolan, L. K., R. E. Wooley, J. Brown, K. R. Spears, H. W. Dickerson, and M. Dekich. Comparison of a complement resistance test, a chicken embryo lethality test, and the chicken lethality test for determining virulence of avian *Escherichia coli*. *Avian Dis.* 36:395–397. 1992.
12. Pfaff-McDonough, S. J., S. M. Horne, C. W. Giddings, J. O. Ebert, C. Doetkott, M. H. Smith, and L. K. Nolan. Complement resistance-related traits among *Escherichia coli* isolates from apparently healthy birds and birds with colibacillosis. *Avian Dis.* 44:23–33. 2000.
13. Sambrook, J., and D. W. Russell. *Molecular cloning: a laboratory manual*, 3rd ed. vol. 1–3. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 2001.
14. Sanderson, K. E., and D. R. Zeigler. Storing, shipping, and maintaining records on bacterial strains. *Methods Enzymol.* 204:248–264. 1991.
15. Southern, E. M. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503–517. 1975.
16. Waters, V. L., and J. H. Crosa. Colicin V virulence plasmids. *Microbiol. Rev.* 55:437–450. 1991.
17. Wooley, R. E., P. S. Gibbs, T. P. Brown, and J. J. Maurer. Chicken embryo lethality assay for determining the virulence of avian *Escherichia coli* isolates. *Avian Dis.* 44:318–324. 2000.