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Pathotyping *Escherichia coli* by Using Miniaturized DNA Microarrays▽†

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The detection of virulence determinants harbored by pathogenic *Escherichia coli* is important for establishing the pathotype responsible for infection. A sensitive and specific miniaturized virulence microarray containing 60 oligonucleotide probes was developed. It detected six *E. coli* pathotypes and will be suitable in the future for high-throughput use.

Pathogenic *Escherichia coli* strains constitute a significant public health problem worldwide (12). In contrast to their nonpathogenic counterparts, these strains have acquired specific virulence attributes that allow them to cause a spectrum of human and animal illnesses (10, 15). Numerous methods exist for the detection of pathogenic *E. coli*, including geno- and phenotypic marker assays for the detection of virulence genes and their products (7, 17, 21, 23). These methods have the common drawback of screening a relatively small number of determinants simultaneously. DNA microarrays offer a viable alternative due to their ability to screen multiple markers simultaneously.

The aim of this work was to develop a simple high-throughput system based in a microtube (details are available from CLONDIAG, Jena, Germany) (13, 20) for pathotyping *E. coli* isolates sent to clinical diagnostic laboratories.

Design and validation of miniaturized virulence arrays. A miniaturized *E. coli* oligonucleotide virulence array was designed containing 39 virulence, 7 bacteriocin, and 15 control (*rrl* and *gad*) gene probes (Table 1). Eighteen genes were specific to a particular *E. coli* pathotype, 13 were common between 2 or more pathotypes, and 7 were unassigned. The design of probes/primers and the specificity were tested as previously described (1, 13).

Control strains were used to validate each probe present on the array (Table 1). PCR amplification and sequencing, using primers given in Appendix 1 of the supplemental material, verified the presence of the probes in control strains. The sequenced genes showed between 92 and 100% sequence identity to the respective target gene and showed 100% sequence identity to the probe and primer regions (data not shown).

Genomic DNA was extracted from cells grown aerobically overnight at 37°C in LB broth, using a DNeasy tissue kit (catalog no. 69504; QIAGEN). One microgram of genomic DNA from each strain was used as a template in a multiplex linear amplification and labeling reaction with the set of 60 primers (Table 1), as previously described (1). The amplified products were added to ArrayTubes for hybridizations performed according to the method of Ballmer et al. (1, 13).

The sequenced strains EDL933, CFT073, and E2348/69 were used to estimate assay sensitivity to ensure strong signal intensity with minimal nonspecific cross-hybridization. Optimization included varying the concentrations of genomic DNA used for labeling (2 to 0.05 μg), the primers present in the linear multiplex mix (0.135 to 0.810 μM), and the poly-horseradish peroxidase–streptavidin conjugate used for detecting hybridization (50 to 400 pg/μl). The minimal concentration of genomic DNA found to reliably detect all expected genes was 1.0 μg, while a concentration of 0.135 μM per primer in the stock solution was sufficient for the detection of target DNA (Fig. 1). The optimal concentration of poly-horseradish peroxidase–streptavidin conjugate was found to be 200 pg/μl; concentrations above or below this value resulted in high background or no detectable reaction at all (data not shown).

The spot signal intensity was derived by calculating the quantitative staining value with IconoClust software (version 2; CLONDIAG). The data were normalized using the signal intensity of the *gad* probe, and the normalized signal intensity for genes within positive and negative control strains was used to differentiate between present (signal intensity value above 0.4) and absent (signal intensity value below 0.3) genes. Genes with signal intensity values between 0.3 and 0.4 were considered ambiguous. Two replicate hybridizations were performed for each control strain, and the 95% confidence interval of error across replicate hybridizations was 1.6 to 3% (see Appendix 2 in the supplemental material).

The specificity of each probe was estimated by comparing array data with PCR and sequenced data from control strains. In all cases, the virulence gene(s) known to be present within positive control strains was clearly identified by array, while two negative control strains, including the sequenced strain MG1655, showed the presence of only 23S rRNA and *gad* genes (see Appendix 2 in the supplemental material). For many positive control strains, additional virulence genes were detected (Table 2). Furthermore, PCR amplification in all

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TABLE 1. Probes and primers used in the miniaturized microarray[a]

Probe/gene	Probe/gene function	Target gene accession no.	Pathotype(s)[b]	Control strain (origin or reference)[c]	Probe sequence (5′–3′)	Primer sequence (5′–3′)
astA	Heat-stable enterotoxin	AE005345.1	EAEC, ETEC	Abbotstown (22)		
astA_11					TCgTgCATATGGTGCgCAACAG	GACGGCTTTGTAgTCCTTCC
astA_21					TCgTgCATATGGTGCgCAACAG	TGACGGCTTTGTAtTCCTTCC
bfpA	Major subunit of bundle-forming pili	AB024946.1	EPEC	E2348/69 (16)	GGTGTGATGTTTTACTACCAGTCTGC	CGCTCATTACTTCTGAAATaGCA
cba	Colicin B pore forming	M16816.1	Undesignated	EC2334/03 (VLA)	GGATGGTCTGTCAGTGTGCACG	GCGGAAACTTTCTCGTTTCC
cdtB	Cytolethal distending toxin B	AJ508930.1	EPEC, STEC, ETEC, ExPEC*	EC934/04 (VLA)		
cdtB_40					GCTGTTGATGCCtTtGGTGGAAG	GCTAACCAGAGCAAGATTGAC
cdtB_50					GCTGTTGATGCCcTtGGTGGAAG	
celb	Endonuclease colicin E2	X03632.1	Undesignated	EC2334/03 (VLA)	GGACCGTATCTCCGTCATCAACAG	GCGTTGCTAATCCGGTCAC
cfaC	Colonization factor antigen I	M55661.1	ETEC	IMI100 (Bern)	GGAATAGCGCGCTGGGTATTACAGA	TCATCCACCAATTTAAGACAGC
cma	Colicin M, resembles beta-lactam antibiotics	M16754.1	Undesignated	EC2334/03 (VLA)	TGTAACGCCGACCGAAATCTGGT	TCATAAACGCTTATTCCAGGGT
cnf	Cytotoxic necrotizing factor	AF483828.1	ExPEC*	S5 (8)	CTTCCAGTATGGGGATCAGTTTTGATCA	CGACGTTCTTCATAAGTATCACC
eae[c]	Intimin	AJ579371.1	EPEC, EHEC	E2348/69 (25)		
eae_10					GTTACAaCaTTATGGAACGGCAGAGGT	CgTCAAAGTTATtACCaCTCTGC
eae_20					GTTACAaCgTTATGGAACGGCAGAGG	AGTcTCGCCAgTATTCgC
eae_30					TGGTgAtAATACCCGtTTAGGtATtGGt	
eae_40[b]					TGGTgAtAATACCCGcTTAGGtATtGG	
f17A[c]	Subunit A of F17 fimbrial protein	AF022140.1	ETEC, ExPEC*	CK210 (6), S5 (8)		
f17A_40[b]					ggTAcTAtGCaACgGgtcaGGC	TGATAAgCGATGGTGTAATTcACaG
f17A_50[b]					CagTAcTAcGCaACgGggtgtGG	TGATAAgCGATGGTGTAATTaACtG
f17A_60[b]					aCaaTAtTAtGCcACaGcgccGG	CTGATAAaCGATGGTGTAATTtACtG
f17G	Adhesin subunit of F17 fimbrial protein	AF022140.1	ETEC, ExPEC*	CK210 (6)	TGCAATGGATAACCTGCCATTTTGTCT	CCAGACATTTGCATTCTGATATCC
fanA	Involved in biogenesis of K99/F5 fimbriae	X05797.1	ETEC	ETEC562 (VLA)	AGCAAGGTGCTTCCAATTATTAGTGGA	CGTAAATACCCCTAGAACTACGT
fasA	Fimbrial 987P/F6 subunit	M35257.1	ETEC	HM1535 (VLA)	GCCAAGTGGATACTTCTAATCTGTCGC	GAGCAGAAGTAGACAACTCTCC
fim41a	Mature Fim41a/F41 protein	X14354.1	ETEC	ETEC562 (VLA)	GGCTTGTTAATCCAGGTCGATTTACTG	GAGAGTCCATTCCATTTATAGGCT
gad	Glutamate decarboxylase	M84025.1	All *E. coli*	All	GATATCGTCTGGGACTTCCGCCT	TGAAGCACTGATCGATTTCACA
ehx (*hlyA*)	Hemolysin A	AB011549.2	EPEC, EHEC	EDL933 (19)	TGTAGGATTAACTGAACGTGGTGTTGC	GCAGAAGTTTGTCAAGTTGTGG
hlyE	Avian *E. coli* hemolysin	AF052225.1	ExPEC*	M1000 (14)	CCAAGATAGATACTTCGAGGCGACAC	TCACTCCACACCATTCATAAACT
*ipaH*9.8	Invasion plasmid antigen	AF047365.1	*Shigella sonnei*	NCTC8192 (HPA)**	TCGCGCTCACATGGAACAATCTC	GCCTGATGGACCAGGAGG
ireA	Siderophore receptor	AF320691.1	ExPEC*	CFT073 (24)	CCACAAATGACTTCTATCTGTCAGGC	CTCCATATAGCTGAAGACCAAGT
iroN	Enterobactin siderophore receptor protein	AF449498.1	ExPEC*	CFT073 (24)	GCCTGTCGAGTAACATGATCAATGCT	GAGGCTTTGCGAAGTGAGC
iss	Increased serum survival	AF042279.1	ExPEC*	CFT073 (24)	CCGCTCTGGCAATGCTTATTACAGG	gGTTTGTTTccAACAGTAAACGT
K88ab	K88/F4 protein subunit gene	V00292.1	ETEC	Abbotstown (22)	GCCTGGATGACTGGTGATTTCAATGG	GTGATACTACCACCGATATCGAC
lngA	Longus type IV pilus	AF004308.1	ETEC	B1308 (VLA)	CGTCTGGTTCATATGCCATGACAGC	CCACAGACATATCTACACCAGT
lthA	Heat-labile enterotoxin A subunit	AB011677.1	ETEC	ETEC21d (VLA)	GGTTTCTGCGTTAGGTGGAATACCA	ACCAAAATTAACACGATACCATCC
mchB	Microcin H47 part of colicin H	AJ515252.1	Undesignated	CFT073 (24)	GGTTGTAGTTGGAGCCGTATCTGC	GGTCGAGCCAATTGCTGT
mchC	MchC protein	AJ515252.1	Undesignated	CFT073 (24)	CTGTCGGGTTAGATCTGTGATCCAC	CCGGTGGTACAGGTAGATATCC
mchF	ABC transporter protein MchF	AJ515251.1	Undesignated	CFT073 (24)	TCCGGTTATTCATCAGACGGAGACC	CAAAATGACCGCATATCATTGC
mcmA	Microcin M part of colicin H	AJ515251.1	Undesignated	CFT073 (24)	CCTCCATGTCTCCCTCAGGTATAGG	GGCACTTGATGTACCTCTGC

Continued on following page

TABLE 1—*Continued*

Probe/gene	Probe/gene function	Target gene accession no.	Pathotype(s)[b]	Control strain (origin or reference)[e]	Probe sequence (5′–3′)	Primer sequence (5′–3′)
perA[c]	EPEC adherence factor, transcriptional activator					
perA_10		AF255772.1	EPEC	E2348/69 (16)	TGTTTGGTTGGGTTTAATTCCACATCA	TTGGTGTTGTGTTGTAATATTCCT
perA_20				N1743-95 (Bern)	GCTTGGTTGGTTTTAATTCCACGTC	
pet	Autotransporter enterotoxin	AF056581.1	EAEC	NZ1470-95 (Bern)	GCTGACAAGGATAATTCTGCCACAAGA	GCATCGCGAGAGCAAACT
prfB/*papB*	P-related fimbriae regulatory gene	X76613.1	ExPEC*	CFT073 (24)	GGGAGACTTATACGGCTGAATGCTC	TCATCTGTATAATAAGGTGCAAGC
senB	Plasmid-encoded enterotoxin	Z54195.1	EIEC	NCTC9774 (HPA)	GCTCTATATCGGACACACCCAGTCAG	GGTGTCAAACATACTGATACGC
sfaS	S fimbria minor subunit	X16664.4	ExPEC*	E536 (VLA)	CAATGCAGGAAGTGGATCTCCATGG	TCCGGTGAGAGACAGATCA
sta1A[c]	Heat-stable enterotoxin ST-Ia					
sta1A_111		AJ555214.1	ETEC	ETEC562 (VLA)	ACACATTTTACTGCTGTGAACTTTGTTG	AACATggAGCACAGGCAG
sta1A_121						AACATccAGCACAGGCAG
sta1B	Heat-stable enterotoxin ST-Ib	AY342058	ETEC	IMI100 (Bern)	AGCAATTACTGCTGTGAATTGTGTTGT	AGCACCCGGTACAAGCAG
stb	Heat-stable enterotoxin II	AJ555214.1	ETEC	Abbotstown (22)	GAGATGGTACTGCTGGAGCATGCT	TTGCTGCAACCATTATTTGGG
stx1A	Shiga toxin 1 A subunit	AB035142.1	STEC	EDL933 (19)	GTGACAGTAGCTATACCACGTTACAGC	TCTGCATCCCCGTACGAC
stx2A	Shiga toxin 2 A subunit	AB035143.1	STEC	EDL933 (19)	GCAGTTATACCACTCTGCAACGTGTC	CtgAttTGCATtCCgGaACG
virF	VirF transcriptional activator, *ipaBCD*-positive regulator	AF386526.1	*Shigella flexneri*	NCTC8192 (HPA)**	GCCTTTTATCAGCTGTTTCTGATGAGGA	GAGAAGAAGCTATCGATATCGAAGT
rrl_0101_0177_10	23S rRNA (large rRNA)	M25458.1	All	E2348/69[d]	GTGTGTTTCGACACACTATCATTAACTGA	GGTTCGCCTCATTAACCTATGG
rrl_0101_0177_20	23S rRNA (large rRNA)	M25458.1	All	E2348/69[d]	GTGTGATTCGTCACACTATCATTAACTGA	
rrl_0260_0330_10	23S rRNA (large rRNA)	M25458.1	All	E2348/69[d]	CAGAGCCTGAATCAGTATGTGTGTTAGT	GCCTTTCCAGACGCTTCC
rrl_0260_0330_20	23S rRNA (large rRNA)	M25458.1	All	E2348/69[d]	GAGCCTGAATCAGTGTGTGTGTTAGT	
rrl_0260_0330_30	23S rRNA (large rRNA)	M25458.1	All	E2348/69[d]	AGAGCCTGAATCAGTTTGTGTGTTAGT	
rrl_0520_0580_10	23S rRNA (large rRNA)	M25458.1	All	E2348/69[d]	GCAGTGGGAGCACGCTTAGG	AAGGTACGCAGTCACACG
rrl_0520_0580_20	23S rRNA (large rRNA)	M25458.1	All	E2348/69[d]	AAGCAGTGGGAGCATGCTTAGG	
rrl_1480_1560_coli_10	23S rRNA (large rRNA)	M25458.1	All	E2348/69[d]	CCGGAAAATCAAGGATGAGGCGTG	CACCGTAGTGCCTCGTCA
rrl_1480_1560_coli_20	23S rRNA (large rRNA)	M25458.1	All	E2348/69[d]	CGGAAAATCAAGGCTGAGGCGTG	
rrl_1480_1560_coli_30	23S rRNA (large rRNA)	M25458.1	All	E2348/69[d]	GGAAAACCAAGGCTGAGGCGTG2	
rrl_1480_1560_shig_40	23S rRNA (large rRNA)	M25458.1	All	E2348/69[d]	GGAAAATCAAGGCCGAGGCGTG	
rrl_1690_1770_coli_10	23S rRNA (large rRNA)	M25458.1	All	E2348/69[d]	GCTGATATGTAGGTGAAGCGACTTGC	CGACTGATTTCAGCTCCACG
rrl_1690_1770_freu_30	23S rRNA (large rRNA)	M25458.1	All	E2348/69[d]	CGCTGATATGTAGGTGAAGTGGTTTACT	
rrl_1690_1770_shig_20	23S rRNA (large rRNA)	M25458.1	All	E2348/69[d]	GCTGATACGTAGGTGAAGCGACTTG	

[a] All probes and primers present in the array representing genes or encompassing allelic variations are listed. The description for each gene, the accession number of the target gene used initially for probe/primer design, the pathotype associated with each gene, and the positive control strain are also given. Probes for the 23S rRNA gene (*rrl*) were included as a species marker, while the *gad* gene was included as an invariant positive control present in low copy number in all *E. coli* strains. *, uropathogenic *E. coli*, avian pathogenic *E. coli*, and neonatal meningitis *E. coli* have been classed together as extraintestinal pathogenic *E. coli* (ExPEC) for this study; **, Health Protection Agency, National Culture Typing Collection. Lowercase letters in sequences indicate sequence variability within the consensus region within which the probe or primer was designed.

[b] EAEC, enteroaggregative *E. coli*; ETEC, enterotoxigenic *E. coli*; EPEC, enteropathogenic *E. coli*; STEC, shigatoxigenic *E. coli*; EHEC, enterohemorrhagic *E. coli*; EIEC, enteroinvasive *E. coli*. "All" indicates all *E. coli*.

[c] Polymorphic genes where different control strains were found to bind to different probe sets or probes showed different signal intensities reflecting allelic variation that had not been distinguished by PCR (see the supplemental material for details).

[d] For details, see www.sanger.ac.uk.

[e] HPA, Health Protection Agency; VLA, Veterinary Laboratories Agency.

control strains of five randomly chosen genes (*eae*, *astA*, *ehx* or *hlyA*, *iss*, and *mcmA*), showed 100% correlation between array and PCR data, indicating the probes to be highly specific with minimum cross-reactions (data not shown).

Pathotyping clinical isolates. A panel of 63 *E. coli* human and animal clinical isolates were pathotyped using the virulence miniaturized microarray (see Appendix 3 in the supplemental material). For five strains, two hybridization reactions

TABLE 2. Virulence determinants detected within each positive control strain

Virulence gene	Gene presence in[a]:																		
	Abbotstown	E2348/69	EC2334/03	EC934/04	IMI100	S5	EDL933	CFT073	CL394	ETEC562	HM1535	M1000	NCTC9774	B1308	ETEC21d	NZ1470–95	E536	N1743–95	NCTC8192
astA	X				X		X									X			
bfpA		X																X	
cba			X								X								
cdtB_40			X	X		X													
cdtB_50			X	X		X													
celb			X																
cfaC					X														
cma			X								X								
cnf			X	X		X													
eae_10		X					X											X	
eae_20		X					X											X	
eae_30		X					X											X	
eae_40		X					X											X	
f17A_40									X										
f17A_50				X															
f17A_60						X													
f17G				X		X			X										
fanA										X									
fasA											X								
fim41a										X									
ehx (*hlyA*)							X												
hlyE												X							
ipaH9.8													X						X
ireA								X				X							
iroN				X		X		X				X					X		
iss		X	X			X		X	X	X	X	X		X			X	X	
K88ab	X															X			
lngA														X					
lthA	X				X										X				
mchB				X				X		X						X			
mchC				X				X		X						X			
mchF				X				X		X		X				X			
mcmA				X				X		X									
perA_10		X															X		
perA_20																			
pet																X		X	
prfB/papB								X				X					X		
senB													X						
sfaS																	X		
sta1A					X					X	X								
sta1B					X														
stb	X																		
stx1A							X												
stx2A							X												
virF																			X

[a] The X's indicate virulence genes present in each positive control strain from using the virulence gene pathoarray, while clear boxes indicate absent genes. The control *rrl* and *gad* genes have been omitted for clarity; see the supplemental material for full details. The mean number of virulence determinants found in the control set of strains was 5, with the highest and lowest numbers being 9 and 1, respectively.

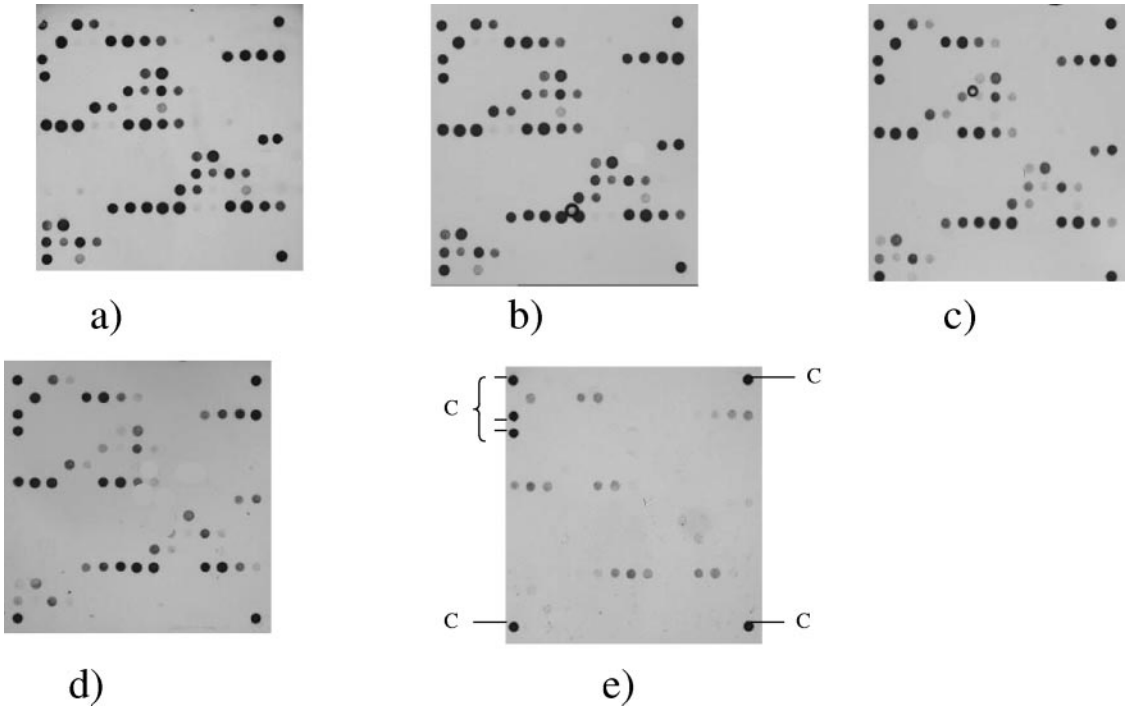

FIG. 1. Optimization of the genomic concentration used in this study. The optimal concentration of genomic DNA from EDL933 used for the detection of genes on the virulence oligonucleotide miniaturized microarray chip was assessed using (a) 2 μg, (b) 1 μg, (c) 0.5 μg, (d) 0.1 μg, and (e) 0.05 μg of DNA. The six biotinylated marker spots (C) are visible in all arrays. A concentration of 0.135 μM per primer in the stock solution and 200 pg/μl of poly-horseradish peroxidase–streptavidin conjugate were used for these assays.

were performed and the 95% confidence interval of error between replicates was 0.9 to 5.0%. Only one hybridization reaction was performed for the remaining test strains.

Fifty-five of the isolates hybridized to more than one virulence determinant and were readily designated within a recognized pathotype, mostly matching the clinical diagnosis where available. Five isolates that harbored only the *iss* gene and/or microcins and three isolates that hybridized to only control genes could not be pathotyped. These isolates may harbor virulence genes not present on our array. Several isolates with novel combinations of genes were detected and included two shigatoxigenic *E. coli* strains, one with *senB*, *iss*, *cma*, *cba*, and *mchBCF* genes and another with *astA*, *cdtB*, and *cnf* genes. The most commonly detected gene was *iss*, which was present in half the strains tested. Other genes which were detected in at least 10 or more isolates included *eae*, *ehx*, *astA*, *iroN*, *mchF*, *mchB*, *mchC*, *f17A* (three variants combined), *f17G*, *mcmA*, *cba*, *cma*, and *prfB*/*papB*. Genes *virF*, *pet*, *hlyE*, *fasA*, and *cfa* were not detected in any test isolate (see Appendix 3 in the supplemental material).

Conclusion. Several *E. coli* virulence arrays for genotyping have been described previously (2–5, 9, 11, 18). These arrays use mostly a glass slide printed with oligonucleotide probes or PCR products for target genes and fluorescent Cy dyes to label DNA used for hybridization. This system is time consuming, with expensive reagents and requires a skilled technician. In contrast, the microtube-based array system used in this study has a short assay time due to an amplification step and inexpensive reagents and requires low technical skills, making it amenable for use in clinical diagnostic laboratories. In the future, the routine use of virulence microarrays in such laboratories will not only allow rapid detection and designation of the pathotypes of strains sent to diagnostic laboratories but also enable emergent strains harboring novel virulence combinations to be detected before such strains spread to become a health problem.

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