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Standard and real-time multiplex PCR methods for detection of trimethoprim resistance *dfr* genes in large collections of bacteria

M. Grape[1], *A. Motakefi*[1], *S. Pavuluri*[1,2] *and G. Kahlmeter*[3]

[1]Department of Microbiology, Tumor and Cell Biology (MTC), Division of Clinical Microbiology, Karolinska Institutet, Stockholm, [2]Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala and [3]Department of Clinical Microbiology, Central Hospital, Växjö, Sweden

ABSTRACT

Two multiplex PCR (mPCR) methods were developed to screen large collections of trimethoprim-resistant *Escherichia coli* isolates for the most prevalent resistance determinants. Five common integron-carried genes (*dfrA1*, *dfrA5*, *dfrA7*, *dfrA12* and *dfrA17*) were selected as PCR targets. Primers and conditions for standard mPCRs and real-time mPCRs were selected and tested. Two protocols using essentially the same primer pairs were established. The standard mPCR protocol also included an internal control targeting the *E. coli* 16S rRNA gene. Both protocols proved to be sensitive and specific for detection of the five selected genes. Screening of three different collections of clinical urinary and blood isolates (n = 368) with the two multiplex methods revealed that the five *dfr* genes accounted for 75–86% of trimethoprim resistance. The standard mPCR is useful and accessible for most laboratories, while the real-time mPCR requires additional equipment and expensive reagents, but is very convenient for high-throughput screening of large collections of bacterial isolates.

Keywords Antibiotic resistance, *dfr* genes, *Escherichia coli*, multiplex PCR, real-time PCR, trimethoprim resistance

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INTRODUCTION

Trimethoprim resistance in clinically significant Gram-negative bacteria is usually caused by horizontally transferable resistance genes (*dfr* genes) coding for alternative resistant dihydrofolate reductases. Most such genes can be found as gene cassettes carried by integrons forming parts of transposons, which mediate widespread dissemination of trimethoprim resistance [1,2]. Of 31 known *dfr* genes, a few types seem to predominate in most parts of the world. In a study of 90 *dfr* gene cassettes carried by class 1 integrons in uropathogenic *Escherichia coli* isolates from 16 European countries and Canada, 36 were *dfrA1*, eight were *dfrA5*, four were *dfrA7*, four were *dfrA12* and 28 were *dfrA17*, with no regional correlation being apparent for this distribution [3]. Furthermore, the same *dfr* types, except *dfrA5*, were identified in 15 non-pathogenic trimethoprim-resistant *E. coli* isolates of food, animal and human origin in Spain [4]. Similarly, studies of gene cassette content in integrons from trimethoprim-resistant Gram-negative urinary isolates from Sweden identified these five genes in 13 of 17 isolates, with two new *dfr* gene types being found in two of the remaining isolates [5]. Other small studies have also suggested that these five genes appear to be the most widespread worldwide [6–10].

Despite these observations, few studies have investigated the distribution of *dfr* genes in large bacterial collections, perhaps because of the absence of a convenient and fast method for screening for these genes. The number of *dfr* gene types, both integron-associated and integron-independent, is growing constantly. Of the known genes, some are very rare or seem to be

Corresponding author and reprint requests: A. Motakefi, Department of Microbiology, Tumor and Cell Biology (MTC), Division of Clinical Microbiology, Karolinska Institutet, L2:02, Karolinska Hospital, SE-171 76 Stockholm, Sweden
E-mail: Alma.Motakefi.9048@student.uu.se

geographically limited. Therefore, it can be difficult to decide which genes to screen for in large collections of isolates. On the basis of the above studies and personal experience, the *dfrA1*, *dfrA5*, *dfrA7*, *dfrA12* and *dfrA17* genes were chosen for the development of two multiplex PCR (mPCR) methods for screening large collections of trimethoprim-resistant *E. coli*.

MATERIALS AND METHODS

Bacteria

Two different collections of bacterial isolates were used to validate the two mPCR methods developed. The first collection (designated UTI-Sto) comprised 105 Gram-negative urinary isolates collected during 2001 by the Department of Clinical Microbiology, Karolinska University Hospital, Stockholm, Sweden, of which 71 were resistant to trimethoprim. Most (n = 75) of these isolates were *E. coli*, while the remainder belonged to various Gram-negative species, most of which were other Enterobacteriaceae. The second collection (designated UTI-Vax) comprised 98 trimethoprim-resistant and 99 trimethoprim-susceptible *E. coli* urinary isolates collected between June and October 2005 at the Department of Clinical Microbiology, Central Hospital, Växjö, Sweden. The UTI-Sto isolates were used to determine the sensitivity of the PCR assays, while the UTI-Vax isolates were used to determine the specificity of the methods. A third collection (designated Blood-Vax) comprised trimethoprim-resistant *E. coli* (n = 66) isolates from blood cultures obtained during the last 20 years at the Department of Clinical Microbiology, Växjö, and was used to further demonstrate the usefulness of the real-time mPCR method.

Preparation of DNA templates for PCRs

All bacterial DNA templates used in PCRs were prepared by boiling approximately three bacterial colonies suspended in 100 μL of sterile filtered water (Sigma-Aldrich, Stockholm, Sweden). The boiled samples were centrifuged briefly and the supernatants were used as DNA templates [5].

Single and standard mPCRs

Amplification reactions (25 μL) contained 1 μL of DNA template, PCR gold buffer (10 mM Tris-HCl, 50 mM KCl) (Applied Biosystems, Stockholm, Sweden), 1.6 mM or 2.5 mM $MgCl_2$ for the single and standard mPCRs, respectively, 0.2 mM dNTPs, pH 7.5 (Amersham Biosciences, Uppsala, Sweden), 0.5 μM each primer (Table 1), 0.6 U of Ampli*Taq*Gold (Applied Biosystems) and sterile filtered water. The primer concentration was doubled for primer dfr7&17-f in the standard multiplex runs. The cycling program comprised 10 min at 95°C, followed by 30 cycles of 45 s at 94°C, 45 s at 50–60°C and 2 min at 72°C, with a final extension for 10 min at 72°C. The annealing temperatures used for the single PCRs were as follows: *dfrA1*, 52°C; *dfrA5*, 55°C; *dfrA7*, 55°C; *dfrA12*, 60°C; and *dfrA17*, 55°C. PCR products were detected by gel electrophoresis (120 V for 40 min) in agarose (Amersham Biosciences) 1% w/v gels stained with ethidium bromide 5 mg/L. The molecular size marker was number VIII (Roche Diagnostics, Bromma, Sweden).

Real-time mPCRs

Reactions (20 μL) contained 1× QuantiTect SYBR Green PCR Kit (Qiagen, Solna, Sweden), 0.3-0.6 μM each primer, RNAse-free water (Qiagen) and 2 μL of template DNA. The real-time PCRs were performed in a Rotor-Gene RG-3000 (Corbett Research, Techtum Laboratory AB, Umeå, Sweden) with Rotor-Gene software v.6.0.25. Following optimisation, each

Table 1. Oligonucleotide primers used for PCRs and sequence analysis

Primer	Locus	Sequence (5′ to 3′)	Product size (bp)	Ref.
dfr1-f	5′-*dfr1*	TGGTAGCTATATCGAAGAATGGAGT	425	This study
dfr1-r	3′-*dfr1*	TATGTTAGAGGCGAAGTCTTGGGTA		
dfr5-f	5′-*dfr5*	AGCTACTCTTTAAAGCCTTGACGTA	341	This study
dfr5-r	3′-*dfr5*	GTGTTGCTCAAAAACAACTTCG		
dfr7&17-f	5′-*dfr7* and 5′-*dfr17*	ACATTTGACTCTATGGGTGTTCTTC	280	This study
dfr7&17-r	3′-*dfr17* and 3′-*dfr7*	AAAACTGTTCAAAAACCAAATTGAA		
dfr7-r	3′-*dfr7*	ACCTCAACGTGAACAGTAGACAAAT	227 with dfr7&17-f	This study
dfr17-r	3′-*dfr17*	TCTCTGGCGGGGGTCAAATCTAT	171 with dfr7&17-f	
dfr12-f	5′-*dfr12*	GAGCTGAGATATACACTCTGGCACT	155	This study
dfr12-r	3′-*dfr12*	GTACGGAATTACAGCTTGAATGGT		
16s-f	5′-*16s*	GCGGACGGGTGAGTAATGT	200	[11]
16s-r	3′-*16s*	TCATCCTCTCAGACCAGCTA		
dfr1s-f	5′-*dfr1*	ATGGAGTGCCAAAGGTGAAC	241	This study
dfr1s-r	3′-*dfr1*	TATCTCCCCACCACCTGAAA		
dfr5s-f	5′-*dfr5*	TCATTAATGGCTGCAAAAGC	460	This study
dfr5s-r	3′-*dfr5*	CCTTTTGCCAAATTTGATAGC		
dfr7s-f	5′-*dfr7*	TCTGCAACGTCAGAAAATGG	404	This study
dfr7s-r	3′-*dfr7*	TGCTCAAAAACCAAATTGAAA		
dfr12s-f	5′-*dfr12*	TTTATCTCGTTGCTGCGATG	457	This study
dfr12s-r	3′-*dfr12*	TAAACGGAGTGGGTGTACGG		
dfr17s-f	5′-*dfr17*	GAAAATATCATTGATTTCTGCAGTG	465	This study
dfr17s-r	3′-*dfr17*	TTTTTCCAAATCTGGTATGTATAATTT		

reaction commenced with 15 min at 95°C, followed by 40 cycles of 20 s at 94°C, 20 s at 62°C and 20 s at 72°C, with a final 30 s at 65°C. A melt point analysis was performed after completion of the 40 cycles using the FAM/sybr program (source at 470 nm and detection at 510 nm) with measurement of the fluorescence at the end of each extension step. With two exceptions (see Results), the same primers were used in the real-time mPCR and the standard mPCR method (Table 1).

Controls

Positive controls for the *dfrA1*, *dfrA5*, *dfrA7*, *dfrA12* and *dfrA17* genes consisted of nine *E. coli* isolates and one *Klebsiella pneumoniae* isolate with previously identified and sequenced *dfr* genes, i.e., two isolates for each *dfr* type [5]. Primers specific for an *E. coli* 16S rRNA gene [11] were included in the standard mPCR as an internal control, but this was not achievable for the real-time mPCR. Negative controls containing sterile filtered water instead of DNA template were also included in all batches of PCRs. In addition, all samples were analysed twice with the mPCR protocols. If a discrepancy between the two runs was observed, the assay was repeated a third time.

DNA sequence analysis

PCR templates purified with a Jetquick spin column kit (Genomed; Saveen Werner AB, Malmö, Sweden) were sequenced using an ABI Prism Big Dye Terminator Cycle Sequencing kit v.3.1 (Applied Biosystems) according to the manufacturer's instructions. Sequence analysis was performed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Subsequent nucleotide sequence analysis was performed using Sequencher software v.4.1.4 (Gene Codes Corporation, Ann Arbor, MI, USA) and the BLAST tool available from NCBI (http://www.ncbi.nlm.nih.gov).

RESULTS

Single PCRs for the five *dfr* genes

Separate PCR assays were performed to detect the presence in the UTI-Sto collection of isolates of the five *dfr* genes selected for inclusion in the mPCRs. The identities of the *dfrA7* and *dfrA17* genes were confirmed by two additional PCRs using the specific primers dfr7&17-f, dfr7-r and dfr17-r (Table 1). In total, 19 *dfrA1*, seven *dfrA5*, five *dfrA7*, seven *dfrA12* and 15 *dfrA17* genes were detected (Table 2). In total, 53 *dfr* genes were detected in 71 trimethoprim-resistant isolates; hence, the five genes were responsible for the trimethoprim resistance in 75% of the isolates in the UTI-Sto collection. The results of these five single PCR experiments were taken as the reference standard against which all subsequent assays were compared.

Standard mPCR for the five *dfr* genes

New primers were designed for the standard mPCR, and several sets were tested to formulate a PCR assay specific for the five selected *dfr* genes. Twelve primers specific for the five genes and the *E. coli* 16S rRNA gene [11] were included in the reaction mixture. Following extensive method development and optimisation, two standard mPCR protocols were formulated. In the first standard mPCR method, the *dfrA7* and *dfrA17* genes could not be differentiated (these genes are 91% identical at the nucleotide level); thus *dfrA7* and *dfrA17* could only be differentiated by sequence analysis. Subsequently, new primers were designed for these two genes, which did not interact with the remaining primers in the PCR mastermix, and which yielded products that were differentiated easily following gel electrophoresis. The final protocol contained one common forward primer for both genes (dfr7&17-f), one common reverse primer for both genes (dfr7&17-r), and one reverse primer (dfr17-r) specific for *dfrA17*. Consequently, isolates carrying *dfrA7* yielded two products, one 200-bp

Table 2. Results obtained using the reference standard (RS) single PCR, regular multiplex PCR (mPCR) and real-time mPCR assays

	UTI-Sto isolates (n = 71)			UTI-Vax isolates (n = 98 resistant isolates)		Blood-Vax isolates (n = 66)
Gene	**RS single PCR**	**Regular mPCR**	**Real-time mPCR**	**Regular mPCR**	**Real-time mPCR**	**Real-time mPCR**
dfrA1	19 (36%)	19 (36%)	19 (36%)	33 (39%)	33 (39%)	26 (50%)
dfrA5	7 (13%)	7 (13%)	7 (13%)	18 (21%)	18 (21%)	6 (12%)
dfrA7	5 (9%)	5 (9%)	5 (9%)	2 (2%)	2 (2%)	1 (2%)
dfrA12	7 (13%)	7 (13%)	7 (13%)	6 (7%)	6 (7%)	0
dfrA17	15 (28%)	15 (28%)	15 (28%)	26 (31%)	25 (30%)	19 (36%)
Total	53 (100%)	53 (100%)	53 (100%)	85 (100%)	84 (100%)	52 (100%)
Overall percentage of trimethoprim resistance			75%		86%	79%

amplicon for the positive control, and one 280-bp amplicon corresponding to *dfrA7*. Isolates carrying *dfrA17* yielded an additional 170-bp amplicon specific for *dfrA17*. Thus, the second standard mPCR had the capacity to distinguish between all five *dfr* genes, as well as the internal control gene, without any requirement for sequence analysis. The final reagent concentrations used in this method are listed in Table 3.

The UTI-Sto collection of isolates was analysed with the second, more specific, mPCR, while both mPCR protocols (with additional sequence analysis) were used to screen the UTI-Vax isolates. The standard mPCR identified 19 *dfrA1*, seven *dfrA5*, five *dfrA7*, seven *dfrA12* and 15 *dfrA17* genes among the UTI-Sto isolates, which was consistent with the results obtained using the reference standard method (Table 2). Analysis of the UTI-Vax isolates using the first protocol in combination with sequence analysis identified 33 *dfrA1*, 17 *dfrA5*, two *dfrA7*, six *dfrA12* and 25 *dfrA17* genes. With two exceptions, the same isolates were positive for the same genes according to the second protocol. One isolate was negative for all five genes according to the first protocol and positive for *dfrA5* according to the second protocol, and a second isolate was positive for *dfrA1* according to the first protocol and positive for *dfrA1* and *dfrA17* according to the second protocol.

Overall, 85 *dfr* genes were detected in 84 of the 98 resistant isolates, and no *dfr* genes were identified in the 99 susceptible isolates. Thus, the five selected *dfr* genes accounted for the trimethoprim resistance of 86% of the isolates investigated. The amplicons were all clearly visible on the electrophoresis gels, and no faint or non-specific bands were observed.

Table 3. Final reagent concentrations used in the standard multiplex PCR method with an optimal annealing temperature of 60°C

Reagents	Final concentration
PCR gold buffer	10 mM Tris-HCl, 50 mM KCl
$MgCl_2$	2.5 mM
dNTPs	0.2 mM
dfr1-f	0.5 μM
dfr1-r	0.5 μM
dfr5-f	0.5 μM
dfr5-r	0.5 μM
dfr7&17-f	1.0 μM
dfr12-f	0.5 μM
dfr12-r	0.5 μM
dfr7&17-r	0.5 μM
dfr17-r	0.5 μM
16s-f	0.5 μM
16s-r	0.5 μM
Taq polymerase	0.6 U

Real-time mPCR for the five *dfr* genes

Different annealing temperatures and primer concentrations were evaluated during the development of the real-time mPCR. Two changes from the standard mPCR protocol were required to achieve optimal reaction conditions. First, the primers for the internal control 16S rRNA gene had to be removed, since the SYBR Green PCR system does not allow reliable detection of more than one PCR product from a single DNA sample. Second, the common dfr7&17-r primer was exchanged for the dfr7-r primer, which is specific for *dfrA7* only. This was possible because amplicon melt points are used for identification instead of product size. Mean amplicon melting temperatures were 81.6°C for *dfrA1*, 85.3°C for *dfrA5*, 78.1°C for *dfrA7*, 83.3°C for *dfrA12*, and 75.0°C for *dfrA17*. The final reagent composition used in the real-time mPCR is listed in Table 4.

The real-time mPCR method was used to analyse both collections of urinary isolates. As with the standard mPCRs, the same isolates were positive for the same *dfr* genes, with the exception of one isolate carrying two different *dfr* genes, for which the real-time mPCR detected only one gene, perhaps because competition between the two amplicons in binding SYBR Green resulted in disturbances in fluorescence detection.

Sequence analysis of *dfr* genes

Sequence analysis was performed for 53 isolates carrying *dfr* genes of all five types.

For all isolates positive for *dfrA7* or *dfrA17* (n = 27) in the UTI-Vax collection, the *dfr* type

Table 4. Final reagent composition used in the real-time multiplex PCR with an optimal annealing temperature of 62°C

Reagents	Final composition
2× QuantiTect SYBR Green PCR Kit	10 μL
dfr1-f	0.33 μM
dfr1-r	0.33 μM
dfr5-f	0.33 μM
dfr5-r	0.33 μM
dfr7&17-f	0.33 μM
dfr7-r	0.33 μM
dfr12-f	0.33 μM
dfr12-r	0.33 μM
dfr17-r	0.33 μM

was determined by sequence analysis of the entire gene. The size of the sequenced amplicons was 465 bp for both *dfrA7* and *dfrA17*, since the primers targeted sequences that were identical in both genes. Primers used for the reference standard single PCR method were used for amplification of DNA template and as primers for sequence analysis. The sequences obtained for two *dfrA7* and 25 *dfrA17* genes, respectively, were almost identical, differing at most by four nucleotide bases, and matched exactly with several published sequences in the EMBL/NCBI databases for these two *dfr* genes.

In the UTI-Vax collection of isolates, the first ten amplicons identified as *dfrA1* and *dfrA5*, and all six *dfrA12* amplicons were also sequenced to validate the specificity of the method. The primers from the reference standard method were used for amplification and sequencing, except for the *dfrA1* amplicons, for which the multiplex primers were used to achieve a larger product. Sequence analysis revealed that all three genes had been identified correctly by the multiplex PCRs, with no differences in the nucleotide sequences obtained, with the exception of 3 bp for *dfrA1*. The sequences obtained for these three genes were identical to a large number of sequences contained in the EMBL/NCBI databases.

Sensitivity and specificity

The sensitivity of the second standard mPCR protocol was 100% in comparison with the reference standard method used to examine the UTI-Sto isolates. For the UTI-Vax isolates, the first and second standard multiplex protocols yielded the same results, except that the second protocol detected two additional genes. When the real-time mPCR was compared with the reference standard method for the UTI-Sto isolates, and with the regular protocol for the UTI-Vax isolates, the sensitivity was 100% and 99%, respectively, with the lower figure explained by the inability to detect two concurrent genes in the same isolate.

The specificity of both the standard mPCR and the real-time mPCR methods was 100%. No *dfr* genes were detected in the 99 trimethoprim-susceptible isolates, and no false-positive results were revealed by sequence analysis of 54 *dfr* genes detected among the UTI-Vax isolates, or in the comparison of results obtained with the regular and real-time protocols for any of the isolates. Thus, although these isolates may harbour other *dfr* genes or trimethoprim resistance mechanisms, the methods described in the present study are specific and do not detect *dfr* genes other than the five selected for the study.

Use of real-time mPCR for screening *E. coli* isolates from blood cultures

The fast and user-friendly real-time mPCR protocol was used to screen 66 trimethoprim-resistant *E. coli* isolates from blood cultures for the five *dfr* genes. Of these isolates, 52 carried one of the five *dfr* genes, comprising 26 *dfrA1*, six *dfrA5*, one *dfrA7* and 19 *dfrA17* genes, accounting overall for 79% of the trimethoprim resistance observed in these isolates.

DISCUSSION

This study describes the development of two alternative mPCR methods for the detection of five of the most common *dfr* genes causing trimethoprim resistance in *E. coli*. The results obtained from testing the methods with a mixed collection of Gram-negative bacteria indicate that these genes are present in most Gram-negative bacteria, and most probably in all members of the Enterobacteriaceae, since *dfr* genes are commonly found as integron gene cassettes in all of these species. The two mPCR protocols formed robust, sensitive and specific detection methods for the five selected genes, and yielded almost identical results with two different collections of bacterial isolates, despite the fact that some of the *dfr* genes are very closely related, e.g., *dfrA7* and *dfrA17*.

The development of two equally reliable methods, using essentially the same set of primers and reaction conditions, makes it possible to choose the method most suitable for particular laboratory conditions. The aim was to make these methods as simple as possible; thus, boiling was used for DNA template preparation rather than expensive kits and robots. The standard mPCR protocol can be performed conveniently in most laboratory environments, while the real-time mPCR requires more advanced and expensive equipment and reagents. However, the latter approach makes the screening of large collections of bacteria for specific trimethoprim resistance genes even more

feasible. The real-time mPCR is quicker, as DNA amplification and melt point analysis requires only 1.5 h.

Initially, primers optimal for the detection of each gene were tested separately with the UTI-Sto collection of isolates. Since the results agreed with the subsequent results using the two mPCR methods, it is probable that these results reflect the true frequency of *dfr* genes in the UTI-Sto collection. One advantage of the standard mPCR protocol is the inclusion of an internal control that detects the *E. coli* 16S rRNA gene. With this control, the integrity of the DNA template can be assured, although no false-negative results caused by an inadequate template were observed. Unfortunately, the real-time protocol did not allow the inclusion of an internal control. External controls, in the form of isolates positive for each of the genes that the method was designed to detect, cannot replace an internal control, but were always included as controls for each batch of reaction mixtures.

Another drawback with a real-time mPCR utilising SYBR Green detection is that it is not possible to reliably identify multiple *dfr* genes in the same isolate. However, it is a rare occurrence for isolates to carry more than one of the five *dfr* genes studied, and this limitation can probably be accepted after taking into consideration the time saved.

The frequencies of the five genes were comparable in the two collections of urinary isolates used for validation (Table 2). In the UTI-Sto collection of mixed Gram-negative isolates, 75% of the trimethoprim-resistant isolates carried one of the five *dfr* genes studied, with a predominance of *dfrA1* and *dfrA17*. In the UTI-Vax collection, consisting of only trimethoprim-resistant *E. coli*, 86% of the resistance was conferred by one of the five *dfr* genes. These results are broadly in line with a large European–Canadian study, in which 108 (66%) of 163 trimethoprim-resistant isolates carried a class 1 integron with one of these five *dfr* genes as an inserted gene cassette [3]. When the third collection of 66 consecutive trimethoprim-resistant *E. coli* blood culture isolates collected during the last 20 years was screened with the real-time mPCR method, the results also indicated that most of the resistance was caused by *dfrA1* and *dfrA17*. The complete absence of *dfrA12* genes was somewhat unexpected, although this gene has not been very prevalent in previous studies.

In conclusion, both mPCR methods proved to be useful tools for detection of the most prevalent trimethoprim resistance genes, namely *dfrA1*, *dfrA5*, *dfrA7*, *dfrA12* and *dfrA17* [3–10]. This is especially important in the case of trimethoprim, since there are no phenotypic methods to indicate which particular gene is conferring resistance. Variations in *dfr* gene distribution may occur as a result of changes in the selective pressure, and screening of this type would provide a sensitive and convenient method for detecting possible changes in the prevalence of different *dfr* genes.

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