The changing nature of aminoglycoside resistance mechanisms and prevalence of newly recognized resistance mechanisms in Turkey

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Objective To determine the most frequently occurring individual and combined resistance mechanisms in Gram-negative bacteria resistant to any of the clinically available aminoglycosides in Turkey, and to compare these mechanisms with those found in smaller, earlier studies.

Methods Aminoglycoside resistance mechanisms in Gram-negative isolates resistant to either gentamicin, tobramycin, netilmicin or amikacin collected in different regions of Turkey were evaluated both phenotypically and genotypically using 12 aminoglycosides and up to 22 aminoglycoside resistance gene probes.

Results Among 696 aminoglycoside-resistant Gram-negative bacteria, resistance rates were very high for gentamicin (94.5%), tobramycin (82.4%), netilmicin (53.6%), and amikacin (49.7%). Although isepamicin was the most active aminoglycoside against Gram-negative bacteria, increased resistance (29.7%) was found and resistance rates were higher than those in most of the other countries surveyed in earlier studies. The most common aminoglycoside resistance mechanisms (AAC(3)-II (GTN), AAC(6′)-I (TNA), and ANT(2″)-I (GT)) in the earlier studies were also found in the present isolates of *Klebsiella* spp., *Enterobacter* spp. and *Escherichia coli*, with increased complexity. In addition to these old mechanisms, two new aminoglycoside resistance mechanisms, namely AAC(6′)-III (TNAI) and AAC(6′)-IV (GTNA), were also found at significant frequencies (11.9% and 26.9%, respectively) in these isolates of Enterobacteriaceae ($n = 435$). Among the isolates of *Pseudomonas* spp. ($n = 150$), in addition to the increased complexity of enzymatic resistance mechanisms (AAC(3)-I (16.6%), AAC(6′)-II (29.3%), AAC(6′)-III (19.3%), ANT(2″)-I (40%)), permeability resistance seemed to be responsible for the high rates of resistance to aminoglycosides.

Conclusion The results of this study indicated increased resistance to clinically available aminoglycosides, including isepamicin, even though it was the most active, as a result of both the presence of new aminoglycoside resistance mechanisms and the increased complexity of all mechanisms, including permeability resistance, particularly in *Pseudomonas* in Turkey.

Keywords Aminoglycoside resistance mechanisms, phenotyping, genotyping, Gram-negative bacteria

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INTRODUCTION

Resistance to the gentamicin and kanamycin families of aminoglycosides in Gram-negative bacteria is most often mediated by a variety of different enzymes that acetylate aminoglycoside acetyltransferase (AAC), adenylate aminoglycoside nucleotdyltransferase (ANT) or phosphorylate aminoglycoside phosphotransferase (APH) aminoglycoside molecules [1,2]. Changes in permeability also play a prominent role in aminoglycoside resistance in *Pseudomonas* [3,4]. Recent publications suggest that permeability resistance in *Pseudomonas* may be largely due to overexpression of an efflux pump, MexXY–OprM, and not to changes in the uptake of aminoglycosides [5,6]. Aminoglycoside-modifying enzymes act on specific sites of aminoglycosides, and the various known sites of modification of the kanamycin and gentamicin families of aminoglycosides are shown in Figure 1. In contrast to enzymatic modification, permeability resistance is not specific and causes decreased susceptibility to all aminoglycosides [4].

Early studies of the prevalence of aminoglycoside resistance mechanisms in Turkey and other countries have documented the occurrence of specific individual mechanisms in Gram-negative bacteria [3,7–9]. In these studies, the incidence of

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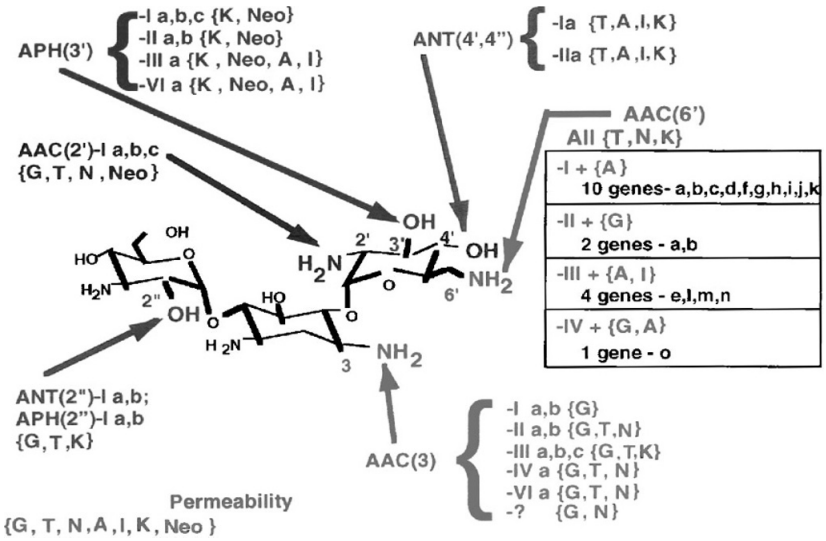

Figure 1 The sites of acetylation (AAC), adenylation (ANT) and phosphorylation (APH) of aminoglycoside molecules by modification enzymes and the genes encoding these enzymes. Inactivation sites of the clinically useful aminoglycosides (gentamicin (G), tobramycin (T), netilmicin (N), amikacin (A), isepamicin (I) or kanamycin (K)) are indicated for each enzyme.

combined resistance mechanisms was very low. In 1985, the most frequent individual mechanisms were ANT(2″)-I, AAC(3)-I, and AAC(3)-II, while only 4.2% of the isolates were resistant due to AAC(6′)-I in Turkey [9]. During this time, the most common individual mechanisms in Enterobacteriaceae were ANT(2″)-I in the USA, AAC(3)-II in Europe, and AAC(6′)-I in Japan [10,11]. During the late 1980s, in addition to the well known combinations of AAC(3)-II and AAC(6′)-I, other combinations began to occur in the USA and Europe [10]. The results of large surveys performed between 1988 and 1993 showed an increased incidence in the complexity of aminoglycoside resistance mechanisms in Latin America, Greece and Turkey, while resistance mechanisms remained less complex in Europe and the Pan-Pacific [12,13]. These surveys also found increased complexity of aminoglycoside resistance mechanisms in *Pseudomonas*.

The aim of this multicenter study was to determine the most frequently occurring individual and combined resistance mechanisms in Gram-negative bacteria that were resistant to any of the clinically available aminoglycosides in Turkey, and to compare these mechanisms with those found in smaller, earlier studies. In addition, we determined the resistance rates of the isolates to clinically available aminoglycosides, including isepamicin, which was not on the market in Turkey during the study period.

MATERIALS AND METHODS

Clinically significant Gram-negative isolates resistant to either gentamicin, tobramycin, netilmicin or amikacin were collected by investigators in 15 hospitals from different geographic regions of Turkey during the first 4 months of 1996. Strains re-isolated from the same patient were not included. Aminoglycoside resistance mechanisms of these aminoglycoside-resistant isolates were determined by using both phenotypic and genotypic methods [14,15]. The phenotypic method, a standardized disk diffusion test, used in the determination of aminoglycoside resistance mechanisms was performed in each hospital. The investigator recorded the preliminary aminoglycoside resistance mechanisms of the isolates according to their relative resistance profiles to 12 aminoglycosides (apramycin, fortimicin, 6′-*N*-ethylnetilmicin, 2′-*N*-ethylnetilmicin, gentamicin, tobramycin, amikacin, isepamicin, netilmicin, 5-episisomicin, kanamycin and neomycin) provided by the Schering-Plough Research Institute (SPRI), New Jersey, USA [14,15]. For genotypic determination of aminoglycoside resistance mechanisms, gene screen hybridization membranes (nitrocellulose paper filters) were inoculated with broth cultures of the isolates. These membranes were processed with 0.5 M NaOH and 1 M Tris (pH 7.0) for binding of bacterial DNA to the filter by the individual investigators in each hospital and then submitted to the SPRI for dot-blot hybridization assay. These filters were incubated with 22 labeled (^{32}P) gene probes (*ant(2″)-Ia*, *ant(4′)-Ia*, *aac(2′)-Ia*, *aac(3)-Ia*, *aac(3)-Ib*, *aac(3)-IIa*, *aac(3)-IIb*, *aac(3)-VIa*, *aac(6′)-Ib*, *aac(6′)-Ic*, *aac(6′)-If*, *aac(6′)-IL*, *aac(6′)-Im*, *aac(6′)-In*, *aac(6′)-Io*, *aac(6′)-IIb*, *aph(3′)-Ia*, *aph(3′)-IIa*, *aph(3′)-IIIa*, *aph(3′)-VIa*, *aph(2″ + 6′)*, rRna) under optimal hybridization conditions and overlayed with X-ray film for autoradiographic detection [15].

Finally, all aminoglycoside resistance mechanisms were assigned by a single investigator (George H. Miller) on the basis of the correlation between the two tests [15]. The data have been analyzed by grouping the isolates as *Klebsiella–Enterobacter–Escherichia coli* (KEE) and *Pseudomonas*, based on the similarity of resistance rates and mechanisms. The remaining isolates, which included *Citrobacter*, *Serratia*, *Salmonella–Shigella*,

Table 1 Aminoglycoside resistance rates in Gram-negative bacteria resistant to any of the clinically available aminoglycosides

Isolates	Number of isolates	Percentage resistance to				
		Gentamicin	Tobramycin	Netilmicin	Amikacin	Isepamicin
Group: KEE						
Klebsiella	221	89.5	98.1	77.3	66.3	12.7
Enterobacter	121	87.6	96.6	52.8	33.8	19.8
Escherichia coli	93	90.3	93.5	47.3	34.4	22.5
Group: *Pseudomonas*						
Pseudomonas	150	98.6	84.6	71.3	58.6	62
Group: Miscellaneous						
Proteus–Providencia	29	96.5	58.6	31.0	20.7	24.1
Serratia	26	96.1	80.8	26.9	7.7	7.7
Citrobacter	16	100	100	62.5	50	0
Salmonella–Shigella	8	100	100	75	62.5	25
Acinetobacter	20	95	45	50	80	65
Stenotrophomonas	12	91.7	66.6	41.7	83.3	58.3
Total	696	94.5	82.4	53.6	49.7	29.7

Proteus–Providencia, *Acinetobacter*, *and Stenotrophomonas*, were evaluated in a separate group named the 'miscellaneous group'.

RESULTS

In this survey, 706 Gram-negative bacterial strains were tested, and 10 of these isolates for which phenotyping and genotyping methods did not agree were excluded. The correlation of the two methods was 98.6%. In this report, aminoglycoside resistance mechanisms of 696 isolates were evaluated. The distribution of bacteria and their resistance rates to aminoglycosides are shown in Table 1.

The most frequent aminoglycoside resistance mechanisms in *Klebsiella, Enterobacter* and *Escherichia coli* (KEE)

Among 435 aminoglycoside-resistant isolates of KEE, 14 different aminoglycoside resistance mechanisms were found. Five of them (ANT(2″)-I (GT), AAC(3)-II (GTN), AAC(6′)-I (TNA), AAC(6′)-IV (GTNA), AAC(6′)-III (TNAI)) and their combinations with both each other and other less frequently seen aminoglycoside resistance mechanisms were found in 96.3% of the isolates (Table 2). Among these mechanisms, AAC(6′)-III and AAC(6′)-IV were newly recognized aminoglycoside resistance mechanisms. The prevalence of common aminoglycoside resistance mechanisms showed differences between *Klebsiella*, *Enterobacter* and *Escherichia coli* (Figure 2). AAC(6′)-IV, one of the two new enzymes, was the most common aminoglycoside-modifying enzyme (37.5%) in *Klebsiella*, whereas ANT(2″)-I (46.2%) and AAC(3)-II (58%) were the most common ones in *Enterobacter* and *Escherichia coli*, respectively, either as single or combined mechanisms (Figure 2, Table 2).

In this group, 52% of the isolates had a single aminoglycoside resistance mechanism. The most frequently occurring single aminoglycoside resistance mechanism was AAC(6′)-IV for *Klebsiella* (35.3%) and *Enterobacter* (16.5%). In *Escherichia coli*, AAC(3)-II was the most common (32.2%) single mechanism (Table 2).

Two hundred and nine (48%) of 435 isolates of KEE had two or more aminoglycoside resistance mechanisms. At least two of the five common aminoglycoside resistance mechanisms (AAC(3)-II, AAC(6′)-I, AAC(6′)-III, AAC(6′)-IV, ANT(2″)-I)

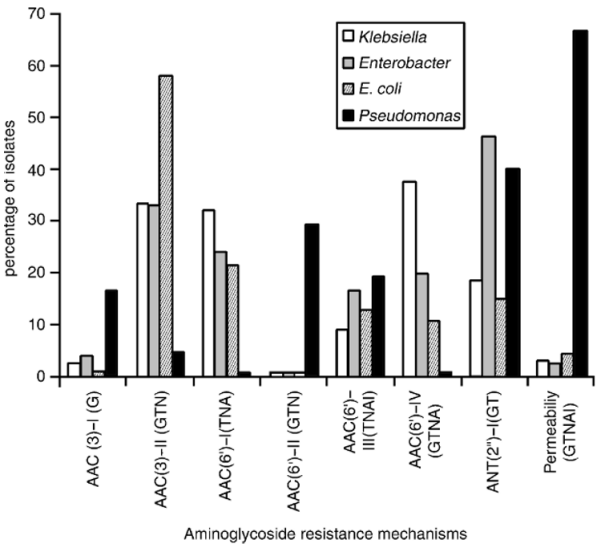

Figure 2 The prevalence of aminoglycoside resistance mechanisms in Gram-negative bacteria.

Table 2 The prevalence of the most common single and combined aminoglycoside resistance mechanisms in *Klebsiella*, *Enterobacter* and *Escherichia coli*

Single or combined aminoglycoside resistance mechanism	Phenotype	*Klebsiella*, $n=221$ (%)	*Enterobacter*, $n=121$ (%)	*Escherichia coli*, $n=93$ (%)	Total
AAC(3)-II (A2)	GTN	9 (4)	12 (9.9)	30 (32.2)	51 (11.7)
AAC(6′)-I (B1)	TNA	5 (2.3)	3 (2.5)	3 (3.2)	11 (2.5)
AAC(6′)-III (B3)	TNAI	4 (1.8)	8 (6.6)	1 (1.1)	13 (3)
AAC(6′)-IV (B4)	GTNA	78 (35.3)	20 (16.5)	10 (10.7)	108 (24.8)
ANT(2″)-I (C1)	GT	14 (6.3)	16 (13.2)	3 (3.2)	33 (7.6)
Permeability resistance	GTNAI	0 (0)	0 (0)	4 (4.3)	4 (0.9)
A2 + B1	GTNA	30 (13.5)	2 (1.7)	4 (4.3)	36 (8.3)
A2 + B1 + B3	GTNAI	3 (1.4)	0 (0)	2 (2.2)	5 (1.1)
A2 + B1 + C1	GTNA	4 (1.8)	3 (2.5)	1 (1.1)	8 (1.8)
A2 + B1 + B3 + C1	GTNAI	0 (0)	2 (1.7)	1 (1.1)	3 (0.7)
A2 + B3 + C1	GTNAI	0 (0)	1 (0.8)	0 (0)	1 (0.2)
A2 + B3	GTNAI	12 (5.4)	2 (1.7)	6 (6.5)	20 (4.6)
A2 + C1	GTN	3 (1.4)	11 (9)	0 (0)	14 (3.2)
A2 + X[a]	–	13 (5.9)	7 (5.8)	10 (10.7)	30 (6.9)
B1 + B3	TNAI	0 (0)	1 (0.8)	1 (1.1)	2 (0.4)
B1 + B3 + C1	GTNAI	1 (0.5)	2 (1.7)	0 (0)	3 (0.7)
B1 + C1	GTNA	15 (6.8)	14 (11.6)	7 (7.5)	36 (8.3)
B1 + X[b]	–	13 (5.9)	2 (1.7)	1 (1.1)	16 (3.7)
B3 + B4	GTNAI	0 (0)	1 (0.8)	0 (0)	1 (0.2)
B3 + X[c]	TNAI	0	0	1 (1.1)	1 (0.2)
B4 + X[d]	–	5 (2.3)	3 (2.5)	0 (0)	8 (1.8)
B3 + C1	GTNAI	0 (0)	3 (2.5)	0 (0)	3 (0.7)
C1 + X[e]	–	4 (1.8)	4 (3.3)	4 (4.3)	12 (2.7)
Total of isolates	–	213 (96.4)	117 (96.7)	89 (95.6)	419 (96.3)

[a]Less frequently occurring aminoglycoside resistance mechanisms. *Klebsiella*: with APH(3′)-I in 11 isolates, with APH(3′)-VI in one isolate, and with an unknown mechanism in one isolate. *Enterobacter*: with APH(3′)-I in five isolates, with AAC(3)-I in two isolates.

[b]Less frequently occurring aminoglycoside resistance mechanisms. *Klebsiella*: with APH(3′)-I in eight isolates, with AAC(3)-I in one isolate, with permeability in one isolate, with unknown mechanism in three isolates; *Enterobacter*: with AAC(3)-IV in two isolates.

[c]Less frequently occurring aminoglycoside resistance mechanisms. With APH(3′)-I in one isolate.

[d]Less frequently occurring aminoglycoside resistance mechanisms. *Klebsiella*: with APH(3′)-I in five isolates. *Enterobacter*: with APH(3′)-I in three isolates.

[e]Less frequently occurring aminoglycoside resistance mechanisms. *Klebsiella*: with APH(3′)-I in four isolates. *Enterobacter*: with APH(3′)-I in three isolates, with APH(3′)-VI in one isolate. *Escherichia coli*: with APH(3′)-I in two isolates, with AAC(3)-III in one isolate, and with AAC(3)-III, APH(3′)-I and APH(3′)-VI in one isolate.

occurred in combination with each other in 30.3% of the isolates (Table 2). Double combinations occurred in 35.7%, 32.2% and 28% of *Klebsiella*, *Enterobacter* and *Escherichia coli*, respectively. Although triple and quadruple combinations of aminoglycoside resistance mechanisms were observed in 12.6% of *Klebsiella*, 17.3% of *Enterobacter* and 17.2% of *Escherichia coli*, the percentages of the isolates with quadruple combinations in each group were much lower than those of the isolates with triple combinations.

In general, the enzymes that modify gentamicin family aminoglycosides (AAC(3)-II, ANT(2″)-I) were found in combination with one or more amikacin-modifying enzymes (AAC(6′)-I, AAC(6′)-III). The combination rates of these enzymes were 29.4% for *Klebsiella*, 23% for *Enterobacter*, and 22.6% for *Escherichia coli* (Table 2).

The most frequent aminoglycoside resistance mechanisms in *Pseudomonas*

In 150 isolates of *Pseudomonas*, nine different aminoglycoside resistance mechanisms were determined. Permeability resistance was the most common (66.6%), followed by ANT(2″)-I (40%), AAC(6′)-II (29.3%), AAC(6′)-III (19.3%), and AAC(3)-I (16.6%) (Figure 2.). At least one of these five mechanisms was found in all isolates.

In this group, 41.3% of the isolates had a single aminoglycoside resistance mechanism. The most common single aminoglycoside resistance mechanisms were ANT(2″)-I (21.3%) and permeability resistance (13.3%) (Table 3).

In the isolates that had more than one aminoglycoside resistance mechanism, 25 different combinations were

Table 3 The prevalence of the most common single and combined aminoglycoside resistance mechanisms in *Pseudomonas*

Single or combined aminoglycoside resistance mechanisms	Phenotype	*Pseudomonas, n* = 150
AAC(3)-I (A1)	G	1 (0.7)
AAC(3)-II (A2)	GTN	2 (1.3)
AAC(6′)-II (B2)	GTN	7 (4.7)
AAC(6′)-III (B3)	TNAI	0
ANT(2″)-I (C1)	GT	32 (21.3)
Permeability resistance (D)	GTNAI	20 (13.3)
A1 + A2 + B3	GTNAI	1 (0.7)
A1 + B2 + B3 + D	GTNAI	1 (0.7)
A1 + B2 + D	GTNAI	1 (0.7)
A1 + B3 + D	GTNAI	7 (4.7)
A1 + D	GTNAI	13 (8.7)
A1 + X[a]	G	1 (0.7)
A2 + B2	GTN	1 (0.7)
A2 + B2 + B3	GTNAI	1 (0.7)
A2 + C1	GTN	1 (0.7)
A2 + X[b]	GTNA	1 (0.7)
B2 + C1	GTN	1 (0.7)
B2 + B3 + D	GTNAI	12 (8)
B2 + B3 + C1 + D	GTNAI	1 (0.7)
B2 + C1 + D	GTNAI	3 (2)
B2 + D	GTNAI	16 (10.6)
B3 + C1 + D	GTNAI	2 (1.3)
B3 + D	GTNAI	4 (2.7)
C1 + D	GTNAI	19 (12.7)
C1 + X[c]	GTAI	1 (0.7)
D + X[d]	GTNAI	1 (0.7)
Total	–	150

[a]Less frequently occurring aminoglycoside resistance mechanisms. With APH(3′)-I in one isolate.

[b]Less frequently occurring aminoglycoside resistance mechanisms. With an unknown mechanism in one isolate.

[c]Less frequently occurring aminoglycoside resistance mechanisms. With APH(3′)-VI in one isolate.

[d]Less frequently occurring aminoglycoside resistance mechanisms. With an unknown mechanism in one isolate.

determined. Double, triple and quadruple combinations occurred in 32.6%, 23.3% and 2.6% of the isolates, respectively. Among the 88 isolates (58.7%) that had combinations, only eight isolates were found to have enzymatic resistance mechanisms without permeability resistance (Table 3).

Aminoglycoside resistance mechanisms in the miscellaneous group: *Citrobacter, Serratia, Salmonella–Shigella, Proteus–Providencia, Acinetobacter, Stenotrophomonas*

In this group, 111 aminoglycoside-resistant Gram-negative bacteria were evaluated. The most common aminoglycoside resistance mechanisms are shown in Table 4.

Table 4 The prevalence of aminoglycoside resistance mechanisms in *Citrobacter, Serratia, Salmonella–Shigella, Proteus–Providencia, Acinetobacter,* and *Stenotrophomonas*

Bacteria	*n*	Aminoglycoside resistance mechanisms (%)							
		AAC(3)-I	AAC(3)-II	AAC(6′)-I	AAC(6′)-II	AAC(6′)-III	AAC(6′)-IV	ANT(2″)-I	APH(3′)-VI	Permeability
Citrobacter	16	0	37.5	62.5	0	0	18.8	43.7	0	0
Serratia	26	50	11.5	57.7	0	3.8	11.5	34.6	7.6	0
Salmonella–Shigella	8	25	37.5	25	0	12.5	50	12.5	0	0
Proteus–Providencia	29	6.9	62	13.8	0	0	3.4	20.7	10.3	10.3
Total of the above	79	21.5	37.9	39.2	0	2.5	13.9	29.1	6.3	3.8
Acinetobacter	20	65	0	5	0	5	20	15	55	15
Stenotrophomonas	12	0	8.3	8.3	33.3	0	0	8.3	8.3	75

Among the isolates ($n = 79$) of *Citrobacter*, *Serratia*, *Salmonella–Shigella* and *Proteus–Providencia*, AAC(3)-I, AAC(3)-II, AAC(6′)-I, ANT(2″)-I and one of the new aminoglycoside resistance mechanisms, AAC(6′)-IV, were found to be the most frequently occurring aminoglycoside resistance mechanisms (Table 4). The other new aminoglycoside resistance mechanism, AAC(6)-III, was found only in *Serratia* and *Salmonella–Shigella*. An amikacin-modifying enzyme, AAC(6′)-I, was found in combination with one of the gentamicin-modifying enzymes (ANT(2″)-I or AAC (3)-II) in 38% of the isolates. Although permeability resistance was prevalent [3,10] in *Proteus–Providencia,* this mechanism did not occur in the other genera of this group.

In *Acinetobacter* ($n = 20$), AAC(3)-I, APH(3′)-VI and AAC(6′)-IV were found to be the most frequently occurring aminoglycoside resistance mechanisms (Figure 1, Table 4.). Most of the isolates (80%) had more than one aminoglycoside resistance mechanism. In 55% of the isolates, AAC(3)-I was found in combination with APH(3′)-VI, which modifies both amikacin and isepamicin (Table 4).

In *Stenotrophomonas* ($n = 12$), permeability resistance was found to be the most frequently occurring aminoglycoside resistance mechanism (75%). AAC(6′)-II, the other common enzyme in this group, was found in 33.3% of the isolates (Table 4). The two new aminoglycoside resistance mechanisms, AAC(6′)-III and AAC(6′)-IV, did not occur in this group of bacteria.

DISCUSSION

Among the 696 aminoglycoside-resistant Gram-negative bacteria tested, the resistance rates were high for gentamicin, tobramycin, netilmicin and amikacin (Table 1). Although isepamicin was not in clinical use during the survey period, resistance to this new aminoglycoside was also found and was particularly high in *Pseudomonas.* The resistance rates among aminoglycoside-resistant KEE isolates to the clinically available aminoglycosides were different from those observed in earlier studies in Turkey. In a study carried out in 1985, among 300 gentamicin-resistant Gram-negative bacterial strains, the resistance rates to tobramycin and netilmicin were also high (78.3% and 43%, respectively), and resistance to amikacin was not observed [9]. Between 1974 and 1988, the resistance rates among aminoglycoside-resistant Enterobacteriaceae isolated from different regions of the world, including Japan, the USA and Europe, to the clinically useful aminoglycosides were similar to those seen in the early Turkish study [10]. Later surveys (1988–93) showed higher netilmicin (71.9%) and amikacin (43.1%) resistance in certain parts of the world, including Latin America, South Africa and Europe, with increased usage of netilmicin and amikacin [12,13]. The strains from Turkey evaluated in those surveys were limited in number and from one hospital, and the results were summarized together with those of a larger number of isolates from Greece. Isepamicin was found to be the most active agent against the isolates, and the resistance rate was quite low overall (9.9%) compared to the current rate of 29.7% (Table 1) [13]. In the present survey, aminoglycoside usage data from 15 participating hospitals indicated that amikacin was the most commonly used aminoglycoside, followed by either gentamicin, tobramycin or netilmicin. This usage pattern correlates with the observed resistance rates to gentamicin, tobramycin, netilmicin, and amikacin. Since isepamicin was not in clinical use during the study period, resistance to it indicates the presence of an aminoglycoside resistance mechanism with cross-resistance to the other aminoglycosides, such as permeability resistance or one of the new amikacin–isepamicin-modifying enzymes such as AAC(6′)-III [16].

Although we found persistence of old aminoglycoside resistance mechanisms, found in early surveys, in Turkey, the determination of two new aminoglycoside resistance mechanisms (AAC(6′)-III, AAC(6′)-IV) and the increased complexity of overall aminoglycoside resistance mechanisms in Gram-negative bacteria against clinically available aminoglycosides are the most significant results of our multicenter study [8–10]. The prevalence of commonly seen aminoglycoside resistance mechanisms showed differences within each bacterial group and genus. However, the comparative evaluation of the resistance rates in each group with the aminoglycoside resistance mechanisms in each of those groups indicated that the combinations of aminoglycoside resistance mechanisms at significant frequencies resulted in high-level resistance to clinically available aminoglycosides (Tables 1 and 2).

The new aminoglycoside resistance mechanisms, AAC(6′)-III and AAC(6′)-IV, which modify tobramycin, netilmicin, amikacin, isepamicin (TNAI) and gentamicin, tobramycin, netilmicin, amikacin (GTNA), respectively, were not present at all in the previous studies [8–10,12,13,16]. AAC(6′)-III confers the same resistance phenotype as AAC(6′)-I, except for the addition of isepamicin, and it was found at remarkable levels in most of the groups of bacteria tested (Tables 2 and 4). It seems to have replaced AAC(6′)-I, which was the most common cause of resistance to amikacin in previous surveys [10,13,16]. This mechanism is coded by the gene *aac(6′)-IL*, which has recently been cloned by Bunny et al [17]. The second new aminoglycoside resistance mechanism, AAC(6′)-IV, was also found at elevated levels in all bacterial groups except for *Pseudomonas* and *Stenotrophomonas*. Interestingly, this mechanism mostly occurred alone. AAC(6′)-IV has a phenotype identical to the previously reported combination of AAC(6′)-I (TNA) and ANT(2″)-I (GT), but is encoded by a newly recognized gene *aac(6′)-Io* [18]. This enzyme is very similar to the enzyme AAC(6′)-I encoded by *aac(6′)-Ib* and differs from it by changes in only two amino acids [18].

In group KEE, although the aminoglycoside resistance mechanism that is first in order varied with the genus to which the isolate belonged, five mechanisms (AAC(3)-II, AAC(6′)-I, AAC(6′)-III, AAC(6′)-IV, ANT(2″)-I) and/or their combinations with each other and/or with less frequently occurring aminoglycoside resistance mechanisms were found at similarly elevated frequencies in each genus (Figure 2, Table 2). One of the new enzymes, AAC(6′)-IV, was the most prevalent in *Klebsiella*, and this mechanism was not observed in any combination with the other aminoglycoside resistance mechanisms but in nine isolates only (Table 2). This single mechanism seems to be sufficient for an isolate to be resistant to all clinically available aminoglycosides except for isepamicin. The remaining four common mechanisms in KEE (AAC(3)-II, AAC(6′)-I, AAC(6′)-III, ANT(2″)-I) were mostly found in combination (Table 2). Although the aminoglycoside resistance mechanisms combined with each other may be altered, major combinations between gentamicin-modifying enzymes (AAC(3)-II, ANT(2″)-I) and amikacin- and/or isepamicin-modifying enzymes (AAC(6′)-I and AAC(6′)-III) were found in ~25% of the isolates in each genus of the KEE group (Table 2). In addition to the single aminoglycoside resistance mechanism, AAC(6′)-IV, all of these combinations correlate with the high-level resistance rates against clinically useful aminoglycosides in this group.

Some changes were recorded in the prevalence of the aminoglycoside resistance mechanisms (AAC(3)-II, AAC(6′)-I) determined in the earlier studies. For example, AAC(3)-II, which was found at a low frequency (7.2%) in the earlier study, was found at a much higher frequency (38.6%) among the current isolates (Table 2) [10,12]. On the other hand, the new enzymes, AAC(6′)-IV and AAC(6′)-III, seem to have replaced AAC(6′)-I, which was the most common (82.6%) cause of resistance to amikacin in the early survey [10,12]. The incidence of AAC(6′)-I decreased to 27.6% in the current study. Since the early study only included one hospital in Turkey, it is not clear that differences are due to time or geographic differences; in any case, they are quite large [10].

Despite the occurrence of less complex aminoglycoside resistance mechanisms in *Escherichia coli* than in *Klebsiella* and *Enterobacter*, this study found increased complexity of aminoglycoside resistance mechanisms when compared with earlier studies performed in Turkey (Table 2) [8,9]. While permeability resistance was not significant in *Escherichia coli* (4%), it was higher than in both *Klebsiella* and *Enterobacter.* In addition to permeability resistance, the other mechanisms that cause isepamicin resistance (AAC(6′)-III and APH(3′)-VI) together resulted in higher resistance to isepamicin in *Escherichia coli* than in *Klebsiella*, a novel finding compared to other surveys (Tables 1 and 2) [10,12,13]. Interestingly, among the isolates of *Escherichia coli*, an unusual aminoglycoside resistance mechanism, AAC(3)-IV, was found at increased frequency (5.4%) when compared with earlier studies [10]. AAC(3)-IV causes resistance to gentamicin, tobramycin, netilmicin and apramycin. This mechanism was quite common (10.9% overall) in Belgium, England, France, and Germany, as apramycin is used in animal husbandry in those countries [10]. In contrast, this mechanism was found in only 0.7% of the isolates from Greece and Turkey, where apramycin was apparently not used. It does not seem to be possible to correlate the presence of this mechanism in Turkey with apramycin usage in 1996, since it is still not used in animal husbandry in Turkey. This occurrence of AAC(3)-IV could indicate the spread of the gene from country to country by more traditional means.

Obviously, these single or combined aminoglycoside resistance mechanisms in KEE are responsible for the high resistance rates to clinically useful aminoglycosides seen among these isolates.

Among the aminoglycoside resistance mechanisms seen in *Pseudomonas*, the high rate of permeability resistance was significant and seems to relate to high-level resistance to clinically available aminoglycosides (Tables 1 and 3). Despite the fact that the occurrence of permeability resistance alone was similar to that seen in other countries, except for Japan, Guatemala, Mexico, Venezuela and the USA, the total incidence of this mechanism (66.6%), including combinations, was much higher than in those countries [4]. For example, between 1988 and 1993, the incidence of permeability resistance was approximately 30% in certain European countries except for France, Greece and Turkey [4]. In addition to permeability resistance, the other common aminoglycoside resistance mechanisms (AAC(6′)-II, and ANT(2″)-I) found in earlier studies were also found in the current isolates [4,13,16]. Interestingly, the incidence of AAC(3)-I, which is common (16.6%) in this survey, was very low in other countries, and, in contrast to those countries, AAC(3)-II was found in only 4.7% of the isolates (Figure 2) [4]. Another mechanism, AAC(6′)-I, which is common in Japan, was not found in Turkey nor in other European countries [10]. Although these enzymatic mechanisms and their combinations with each other were found at elevated levels, they were mostly found in combination with permeability resistance. Among the 88 isolates that had combinations, enzymatic mechanisms were found alone in only eight isolates without permeability resistance. As a result of the high incidence of permeability resistance in *Pseudomonas*, the resistance rates against clinically available aminoglycosides were much higher than those seen in *Klebsiella*, *Enterobacter and Escherichia coli* (Table 1).

Despite the low number of isolates in each genus of the miscellaneous group, the aminoglycoside resistance mechanisms and their combinations seen in this group correlate with the high resistance rates against aminoglycosides, including amikacin and isepamicin, in certain genera, particularly in *Acinetobacter* and

Stenotrophomonas. The high prevalence (75%) of permeability resistance in *Stenotrophomonas* and in *Pseudomonas*, and of APH(3′)-VI, which modifies amikacin and isepamicin in *Acinetobacter*, explains this high level of resistance to amikacin and isepamicin (Tables 1 and 4). On the other hand, the high incidence (55%) of the combination between AAC(3)-I and APH(3)-VI, together with AAC(6′)-IV and permeability resistance, explains the high resistance rates against all clinically useful aminoglycosides in *Acinetobacter*. In contrast to the earlier survey, AAC(3)-II was not found in *Acinetobacter* [13]. The aminoglycoside resistance mechanisms seen in the remaining miscellaneous group were similar to those in group KEE, with some changes in their prevalence (Table 4). Despite the occurrence of AAC(6′)-IV in all of these genera, the other new enzyme (AAC(6′)-III) was not found in *Citrobacter* and *Proteus–Providencia*. This result correlates with the absence of isepamicin resistance in *Citrobacter* (Tables 1 and 4). On the other hand, the remarkably high level of permeability resistance (10.3%) in *Proteus–Providencia* seems to be responsible for isepamicin resistance in this group. In *Serratia*, although AAC(6′)-I was common (57.7%), the resistance rate against amikacin remained low (7.7%) (Tables 1 and 4). It is well known that *Serratia* has the chromosomal *aac(6′)-Ic* gene, and the isolates that have the chromosomal gene do not show resistance to amikacin phenotypically [1]. However, the isolates that have the plasmid-derived *aac(6′)-Ib* gene, which is common in other members of the Enterobacteriaceae, show resistance to amikacin phenotypically. This might be the reason for low-level amikacin resistance in this group. All of these mechanisms and the high level of combinations of AAC(6′)-I with one of the gentamicin-modifying enzymes resulted in high-level resistance to all clinically available aminoglycosides.

Finally, the results of this study indicated increased resistance to clinically available aminoglycosides, including isepamicin, even though it was the most active compound, as a result of both the presence of new aminoglycoside resistance mechanisms and increased complexity of all aminoglycoside resistance mechanisms, including permeability, particularly in *Pseudomonas* in Turkey.

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