EVALUATION OF MICROBIOLOGICAL ASSAYS OF ANTIBIOTICS

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Summary

The present study was aimed to assess and compare the potential inhibitory effects of some commonly used antibiotics against five clinically significant fecal coliforms, such as, *Escherichia coli, Salmonella typhi, Proteus vulgaris, Bacillus subtilis* and *Shigella shigae* by *in-vitro* Kirby- Bauer and broth dilution methods. Identity of test organisms was confirmed based on microscopic examination, cultural, colony characteristics and biochemical profile (IMViC tests). Results of the present studies clearly demonstrated that, all the test organisms were proved to be Gram negative except *B. subtilis* is a Gram positive bacterium, however, only *E. coli, B. subtilis* and *P. vulgaris* were observed as motile. The results of the IMViC tests clearly demonstrated that, *E. coli* and *P. vulgaris* were positive to Indole, Methyl red tests, *B. subtilis* was positive to Voges - Proskauer test. Perhaps *S. typhi* showed positive to methyl red and citrate utilization tests. *Sh. Shigae* to Methyl-Red and Voges - Proskauer tests. Based on *Kirby-Bauer* studies it is clear that, *S. typhi* was found to be highly sensitive to penicillin and gentamycin, similarly, *B. subtilis* was sensitive to ampicillin antibiotics, where as, *Sh. shigae* was found to be sensitive to kanamycin. Broth dilution method: here, penicillin was effectively inhibited the growth of all selected pathogenic bacteria from 2 µg/ml, where as, streptomycin inhibited the growth of a majority of these pathogens from 1 µg/ml. Kanamycin was effective to *S. typhi* from 1 µg/ml itself, ampicillin to *B. subtilis* and *P. vulgaris* from 2µg/ml. Gentamycin was effective against *E. coli* and *B. subtilis* from 2 µg/ml itself. From these results, it is clear that, these microbial based natural products still appear as the promising source of the future antibiotics.

Key Words: antibiotics, Kirby-Bauer method, broth dilution method, microbial based antibiotics

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Introduction

Microbial contamination and possible infection of the host are major concerns in the area of the therapeutic medical devices. Ongoing studies are directed toward understanding the mechanism of microbial adsorption and proliferation on material surfaces (1-2). The frequent outbreaks of infectious diseases, which are of bacterial origin, are mainly caused due to *E. coli, S. typhi P. vulgaris, B subtilis* and *Sh. Shigae* (3). Enterobacteriaceae members have been reported as major cause of acquired nosocomial infections including urinary tract infections, intra-abdominal and pelvic infections, endocarditis, pelvic infections, bractemia and meningitis (4).

Although certain microorganisms developed resistance to many antimicrobial agents, including gentamicin and many β-lactams, has become fairly common in recent years. Detailed survey reports from other sources have clearly indicated that, majority of the synthetic drugs are failured at the first step in clinical trials due to high toxicity and other side effects, moreover, plant based antimicrobials have proven low to mild activity. In addition to these and other facts, till to date, almost all the *in-vitro* antibacterial and antifungal research, these microbial based antibiotics are used as a standards, indicating that, still these microbial products remain the most promising source of antibiotics, although new approaches are required to improve the efficiency of these drugs.

By considering these facts, the present study was performed to compare parallel *in-vitro* antibacterial activities of kanamycin, gentamicin, penicillin, streptomycin and ampicillin against few clinically significant fecal coliforms and determined the potency of each antibiotic drug.

Materials and Methods

Antimicrobial agents: The filter paper discs carrying penicillin, streptomycin, kanamycin, ampicillin and gentamicin antibiotic drugs were procured from Difco Laboratories Limited and were stored at 4°C. The solvents and other chemicals used were analytical grade.

Culture Media: The nutrient agar, nutrient broth and Mueller- Hinton Agar [MHA], Mueller- Hinton Broth [MHB], Mac-Conkey agars were purchased from HiMedia, Laboratories Limited, Mumbai.

Microorganisms Used: The organisms employed in the present study were clinical isolates obtained from the standard stock cultures of Microbiology Laboratory, Basaveshwara Hospital, Gulbarga, India.

Kirby-Bauer Method: Susceptibility tests were performed by the disc diffusion method (5) with Mueller Hinton Agar. Zones of inhibition were measured after 24 hours of incubation at 37°C. Simultaneous agar dilution susceptibility tests were performed with the same suspension of bacteria as prepared for the disc test but diluted so that an inoculum replicator would deposit approximately 10^5 viable cells of each strain onto each plate (6).

Broth dilution method: This method was used to determine susceptibility of microbes to precise quantities of an antibiotic. In this method, the test bacterium was inoculated into M.H.B. tubes containing serial dilutions of the antibiotic. The inoculated cultures are incubated for a suitable period of time, that is, 24 hours and the presence or absence of growth was determined by the turbidity in each tube. This method is considered accurate for determining susceptibility of a bacterium to precise quantities of an antibiotic (7)

Statistical Analysis: All the data are expressed as mean ± S.E.M. (standard error of the mean). The significance level was determined using the Student't' test. A *p*-value of <0.05 was considered statistically significant.

Results

Identification of organisms: The results indicated that, *Escherichia coli* are Gram negative, motile, short rods. The colonies of *E. coli* appear large, thick, grayish-white, moist, smooth, opaque and partially translucent on ordinary agar media, showed positive to indole and methyl-red biochemical tests. *Salmonella typhi* is a Gram negative, rod shaped, occurs singly and in pairs and occasionally in short chains. It is non-motile, growing rapidly on simple media. Its colonies are medium size, 2-3 mm in diameter, circular and smooth on Mac-Conkey agar. It has shown positive to methyl-red and citrate production tests. *Shigella shigae* is a Gram negative, short rod, non-motile, non-sporing and non-capsulated. After overnight incubation, colonies are small, about 2mm in diameter, circular, convex, smooth and translucent on normal agar media. Colonies on Mac-Conkey agar are colourless due to the absence of lactose fermentation. It is positive to methyl-red and Voges-Proskauer tests. Where as *Bacillus subtilis* species are identified as Gram positive, small rods occurred singly or in short chains, spore forming and motile. Spores were central with rounded singly or in short chains, spore forming and motile and abundant growth occurred in nutrient agar and positive to only Voges-Proskauer test.

Proteus vulgaris is a Gram negative, motile, rod shaped, emits characteristic fleshy or seminal odour, when grown on a nutrient and blood agar, they exhibit swarming. They also form smooth, pale or colourless colonies on Mac-Conkey agar and do not swarm and showed positive for indole production, urease production, methyl-red and catalase tests (Table -1).

Kirby-Bauer agar diffusion technique: The antibacterial activity of five commonly used antibiotics is summarized in the table – 2 and it is evident that, the selected antibiotics showed antibacterial activity with varying magnitudes. The zone of inhibition above 14 mm in diameter was taken as positive result. Penicillin and streptomycin antibiotics were failured to inhibit any of the tested strains at low doses, where as, same drugs were proved to be effective only at higher concentrations. In contrast to this, ampicillin, kanamycin and gentamicin were effective to almost all test organisms even at mild doses. From the results, it is clear that, gentamicin, ampicillin and kanamycin are superior to penicillin and streptomycin for control of bacterial growth in *in-vitro* studies. It is also clear from the table-2 that, only *S. typhi* was sensitive to penicillin, but remaining four pathogens were proved to be resistant. However, antibiotic drug streptomycin has shown better activity against *S. typhi* and *Sh. shigae*, but mild to moderate activity against *P. vulgaris.* Where as, *E. coli* and *B. subtilis* were found to be resistant. Where as, kanamycin, ampicillin and gentamicin antibiotics were effectively inhibited all the test bacterial growth even at very low doses. .

Broth dilution method: This method is based on the inhibition of growth of a microbial culture in a fluid medium containing a uniform solution of an antibiotic It is evident from the table –3 that, penicillin inhibited the growth of all selected pathogenic bacteria from 2 μg/ml, where as, streptomycin inhibited the growth of a majority of these pathogens from 1 μg/ml. Kanamycin was effective to *S. typhi* from 1 μg/ml itself, ampicillin was effective to *B. subtilis* and *P. vulgaris* from 1μg/ml. Gentamycin was effective against *E. coli* and *B. subtilis* from 2μg/ml and 0.6μg/ml respectively.

Table-1 Microscopic and Biochemical profile of Clinical isolates

Bacteria	**Gram staining**		**Motility**		**IMViC tests**			
	Positive	Negative	Motile	Non-motile	Indole	Methyl Red	Voges Proskauer	Citrate utilization
E. coli	-	+	+	-	+	+	-	-
B. subtilis	+	-	+	-	-	-	+	-
P. vulgaris	-	+	+	-	+	+	-	-
S. typhi	-	+	-	+	-	+	-	+
Sh. shigae	-	+	-	+	-	+	+	-

Note: IMViC – Collection of Indole production, Methyl red, Voges-Proskauer test and Citrate utilization tests.

Table-2 Evaluation of microbial assays of antibiotics using Kirby-Bauer method

Drug	Bacteria	Zone of inhibition (mm)												
		0.2 (µg)	0.4 (µg)	0.8 (µg)	1.0 (µg)	2.0 (µg)	4.0 (µg)	6.0 (µg)	8.0 (µg)	10.0 (µg)	12.0 (µg)	14.0 (µg)	16.0 (µg)	18.0 (µg)
Penicillin	*E. coli*	-	-	-	-	-	-	-	08.20 ±0.34	11.30 ±0.11	14.23 ±0.46	16.00 ±0.20	18.18 ±0.10	21.43 ±0.31
	B. subtilis	-	-	-	-	-	07.75 ±0.35	08.73 ±0.24	10.50 ±0.64	11.41 ±0.15	13.17 ±0.23	15.03 ±0.40	16.05 ±0.41	22. .33 ±0.13
	P. vulgaris	-	-	-	-	07.83 ±0.32	10.50 ±0.64	12.46 ±0.25	15.00 ±0.74	20.20 ±0.09	22.80 ±0.40	24.07 ±0.41	26.04 ±0.81	29.22 ±0.38
	S. typhi	-	-	-	18.05 ± 0.06	19.00 ±0.81	20.25 ±0.85	22.61 ±0.20	25.12 ±0.42	FS				
	Sh. shigae	-	-	-	9.01 ±0.04	13.00 ±0.40	18.25 ±0.85	24.80 ±0.20	26.00 ±0.122	28.90 ±0.21	32.00 ±0.70	38.05 ±0.23	46.25 ±0.47	FS
Streptomycin	*E. coli*	-	-	-	-	-	-	-	09.33 ±0.25	11.60 ±0.12	13.38 ±0.25	16.00 ±0.20	18.17 ±0.13	21.08 ±0.30
	B. subtilis	-	-	-	-	-	07.44 ±0.33	08.90 ±0.44	10.17 ±0.47	11.27 ±0.12	1317 ±0.31	15.07 ±0.54	16.00 ±0.22	22.32 ±0.13
	P. vulgaris	-	-	-	-	07.20 ±0.24	10.40 ±0.23	12.45 ±0.29	15.60 ±0.20	20.20 ±0.09	22.83 ±0.31	24.08 ±0.41	26.46 ±0.18	29.23 ±0.38
	S. typhi	-	-	-	18.00 ±0.31	19.03 ±0.12	20.25 ±0.37	22.26 ±0.44	25.25 ±0.16	FS				

	Sh. shigae	-	-	-	19.00 ±0.20	20.97 ±0.45	22.54 ±0.85	24.58 ±0.20	26.00 ±0.20	28.40 ±0.21	32.00 ±0.19	38.05 ±0.13	44.98 ±0.71	FS
Kanamycin	*E. coli*	-	-	-	30.01 ±0.40	35.50 ±0.64	36.00 ±0.81	37.30 ±0.21	37.25 ±0.25					
	B. subtilis	-	-	-	34.50 ±0.64	36.00 ±0.70	37.75 ±0.95	38.13 ±0.41	37.25 ±0.25	39.20 ±0.11	FS			
	P. vulgaris	-	-	-	32.75 ±0.32	36.00 ±0.81	36.50 ±0.81	39.20 ±0.10	38.90 ±0.36	FS				
	S. typhi	-	-	-	21.25 ±0.63	26.00 ±0.31	30.00 ±0.64	31.12 ±0.51	31.00 ±0.81	FS				
	Sh.. shigae	-	-	-	32.00 ±0.81	33.70 ±0.31	34.00 ±0.41	35.30 ±0.26	35.00 ±0.82	FS				
Ampicillin	*E. coli*	-	-	-	18.75 ±0.85	20.03 ±0.28	21.00 ±1.08	22.47 ±0.18	23.47 ±0.82	25.60 ±0.26	26 ±	27.15 ±0.15	27.13 ±0.55	28.00 ±0.41
	B. subtilis	-	-	-	23.00 ±0.81	27.80 ±1.84	28.09 ±0.98	29.23 ±0.26	30.03 ±0.40	31.12 ±0.51	33 ±	33.35 ±0.23	34.27 ±0.15	FS
	P. vulgaris	-	-	-	17.00 ±0.40	19.25 ±0.08	20.25 ±0.85	20.43 ±0.24	FS					
	S. typhi	-	-	-	19.25 ±0.62	21.85 ±0.38	23.38 ±0.23	24.33 ±0.19	25.25 ±0.35	25.07 ±026	26.25 ±0.37	27.13 ±0.56	28.95 ±0.18	FS
	Sh. shigae	-	-	-	21.00 ±0.20	23.40 ±0.57	25.02 ±0.41	27.38 ±0.22	29.00 ±0.20	30.97 ±0.69	32.88 ±0.43	FS		
Gentamycin	*E. coli*	-	-	-	21.75 ±0.47	23.75 ±0.48	25.00 ±0.49	26.47 ±0.29	27.13 ±0.24	28.25 ±0.25	29.23 ±0.20	FS		
	B. subtilis	-	-	-	-	25.22 ±0.73	27.00 ±0.91	28.25 ±0.24	28.92 ±0.31	30.50 ±0.96	FS			
	P. vulgaris	-	-	-	21.00 ±0.20	29.90 ±0.41	30.75 ±0.48	32.80 ±0.17	33.25 ±0.11	35.00 ±0.41	36.13 ±0.42	FS		
	S. typhi	-	-	-	-	30.00 ±0.50	38.25 ±0.85	40.73 ±0.31	FS					
	Sh. shigae	-	-	-	19.00 ±0.81		22.05 ±0.40	23.45 ±0.16	25.29 ±0.35	26.05 ±0.65	29.93 ±0.19	30.51 ±0.65	31.05 ±0.15	31.00 ±0.41

Note : FS – Fully sensitive

Table-3 Evaluation of microbial assays of antibiotics using broth dilution method

Drug	Bacteria	Concentration (μg/ml)									
		0.2	0.4	0.6	0.8	1.0	2.0	4.0	6.0	8.0	10.0
Penicillin	*E. coli*	+	+	+	+	+	-	-	-	-	-
	B. subtilis	+	+	+	+	+	-	-	-	-	-
	P. vulgaris	+	+	+	+	+	-	-	-	-	-
	S. typhi	+	+	+	+	-	-	-	-	-	-
	Sh. shigae	+	+	+	+	+	-	-	-	-	-
Streptomycin	*E. coli*	+	+	+	+	+	-	-	-	-	-
	B. subtilis	+	+	+	+	-	-	-	-	-	-
	P. vulgaris	+	+	+	+	+	-	-	-	-	-
	S. typhi	+	+	-	-	-	-	-	-	-	-
	Sh. shigae	+	+	+	+	-	-	-	-	-	-
Kanamycin	*E. coli*	+	+	+	+	+	+	-	-	-	-
	B. subtilis	+	+	+	+	+	-	-	-	-	-
	P. vulgaris	+	+	+	+	+	-	-	-	-	-
	S. typhi	+	+	+	+	-	-	-	-	-	-
	Sh.. shigae	+	+	+	+	+	+	+	+	-	-
Ampicillin	*E. coli*	+	+	+	+	+	+	-	-	-	-
	B. subtilis	+	+	+	+	-	-	-	-	-	-
	P. vulgaris	+	+	+	+	+	+	-	-	-	-
	S. typhi	+	+	+	+	+	+	-	-	-	-
	Sh.. shigae	+	+	+	+	+	+	-	-	-	-
Gentamycin	*E. coli*	+	+	+	+	+	-	-	-	-	-
	B. subtilis	+	+	-	-	-	-	-	-	-	-
	P. vulgaris	+	+	+	+	+	+	-	-	-	-
	S. typhi	+	+	+	+	-	-	-	-	-	-
	Sh.. shigae	+	+	+	+	+	+	-	-	-	-

Note: "+" indicates turbidity, and "-" indicates inhibition of growth

Discussion

The clinical significance of *E. coli, S. typhi, Sh. Shigae, B. subtilis* and *P. vulgaris is* a strong factor for regular monitoring of their sensitivity to both established and novel compounds. Hence, these human isolates were collected from different pathological sources and tested for their sensitivity to gentamicin, ampicillin, streptomycin, kanamycin and penicillin antibiotics. The results revealed that, the selected antibiotics showed antibacterial activity with varying magnitudes. From these results it is clear that these antibiotics effectively inhibited the bacterial growth in *in-vitro* studies. The effectiveness of these drugs in *in-vivo* condition was confirmed through the survey reports, as well as usage of these in our day to day life. The reduction in growth rate was linearly related to concentration of antibiotic. Among different antibiotics studied, only kanamycin, gentamicin and ampicillin were proven most effective and superior over the penicillin and streptomycin antibiotics. The effectiveness of gentamicin and kanamycin were also highlighted by several reports. Kanamycin is a water soluble aminoglycoside antibiotic and is active against *Pseudomonas aeruginosa* (8). Gentamicin is a polycationic aminoglycoside antibiotic with broad spectrum antibacterial activity. Its use is indicated in several serious bacterial infections requiring hospitalization. The aminoglycosides are freely soluble in water, and after intravenous and intramuscular administration. (9). Fountain *et al.,* (1985) (10) found enhanced antimicrobial activity with EPC-encapsulated gentamicin in the treatment of infections caused by the intracellular pathogen *Brucella* spp. both *in-vitro* and *in-vivo.* Other Egg phosphatidylcholine (EPC) and EPC-cholesterol based formulations have been used to treat infections caused by *Salmonella dublin* (11), and growth inhibition experiments have shown that liposomal gentamicin has enhanced efficacy over that of free gentamicin against *E. coli* and *P. aeruginosa* infections (12). *Mycobacterium avium* infections in human AIDS patients have also been treated with liposomal gentamicin with some success (13). Recently, focus on antimicrobial potential of microbial based antibiotics in the hope that one day it will be possible to find suitable pharmacological agents/ that could protect entire human race against the serious effects of microbial infections and history of antimicrobial efficacy of microbial sources repeats once again (14)

Conclusion

In summary, the results presented here demonstrate that, penicillin, gentamicin, streptomycin, kanamycin and ampicillin were compared in parallel tests *in-vitro* against a variety of bacterial strains. A number of differences were seen in particular the lower

activity of penicillin and streptomycin and greater activity of kanamycin, ampicillin and gentamicin against test organisms. Detailed survey of antimicrobial research from other sources indicated that, there are disadvantages using plants as antimicrobial agents, such as: low to mild efficacy, however, synthetic compounds have proven as highly toxic at optimum therapeutic doses. By considering the above facts, it is concluded that, Still, microbial natural products remain the most promising source of novel antibiotics because of their relatively high tentative specific activity. Although, novel approaches are required to improve the efficacy of drugs.

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