Occurrence and antibacterial susceptibility pattern of bacterial pathogens isolated from diarrheal patients in Pakistan

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ABSTRACT

الأهداف: لتحديد حدوث مسببات الأمراض البكتيرية المسؤولة عن الإسهال وتزويد المعلومات بشأن فعالية المضادات الحيوية المستخدمة عادة ضد الإسهال.

الطريقة: أجريت هذه الدراسة مستعرضة خلال الفترة ما بين أبريل ويوليو 2014. جمعت عينات من شعبة المقر الرئيسي Allied Hospital، فيصل آباد، باكستان. استخدم الأوساط الغذائية التفريقي والإنتقائي لعزل مسببات الأمراض البكتيرية، التي تم تحديدها من خلال الخصائص الثقافية والفحص المجهري والاختبارات البيو كيميائية. وأجري فحص القرص نشرها خارج باستخدام أغار مولر هنتون المتوسطة، وتحديد الحد الأدنى لتركيز مثبط باستخدام broth dilution ضد مسببات الأمراض معزولة.

النتائج: كانت مائة وإحدى وأربعين (100%) عينة إيجابية لبعض أنواع البكتيريا. وكان تواتر حدوث العصوية الشمعية (*B. cereus*) (66%)، الإشريكية القولونية (*E. coli*) (48.5%) السَّلْمونيلَة التِّيفيَّة (*S. Typhi*) (27.7%)، الزائفة الزنجارية (*P. aeruginosa*) (8.5%)، والمكورات العنقودية الذهبية (*Staph. aureus*) (4.3%). تم الكشف عن العوامل المسببة للأمراض واحد في 20 (14.2%) عينة بينما تم العثور على مجموعات في 121 عينة (85.8%). كان *B. cereus* و *E. coli* من أكثر المسببات للأمراض تليها *S. Typhi* ، *Staph. aureus* ، *Staph. aureus*. وكان وقوع نسبة مسببات الأمراض معزولة 31% في *B. cereus* ، و 31% في *E. coli* ، و 18% في *S. Typhi* ، 5% في *P. aeruginosa* ، و 3% في *Staph. aureus*.

الخاتمة: أظهر الزائفة الزنجارية المقاومة ضد أموكسيسيلين وسيفوتاكسيم في حين تم العثور على *Staph. aureus* مقاومة ضد سيفوتاكسيم. وكشف التحليل الإحصائي باستخدام تحليل التباين الأحادي أن أوفلوكساسين وجنتاميسين كان معنوياً ($p>0.05$) ضد كل يعزل بالمقارنة مع المضادات الحيوية الأخرى المستخدمة في هذه الدراسة.

Objective: To determine the occurrence of bacterial pathogens responsible for diarrhea and to engender information regarding the effectiveness of commonly used antibiotic against diarrhea.

Methods: This cross-sectional study was conducted between April and July 2014. Samples were collected from the Divisional Headquarter and Allied Hospital, Faisalabad, Pakistan. The differential and selective media were used to isolate bacterial pathogens, which were identified through cultural characteristics, microscopy, and biochemical tests. Disc diffusion assay was carried out using Muller Hinton agar medium, and minimum inhibitory concentration was determined using broth dilution method against isolated pathogens.

Results: One hundred and forty-one (100%) samples were positive for some bacteria. Frequency of occurrence was *Bacillus cereus* (*B. cereus*) (66%), *Escherichia coli (E. coli)* (48.5%), *Salmonella typhi (S. Typhi)* (27.7%), *Pseudomonas aeruginosa (P. aeruginosa)* (8.5%), and *Staphylococcus aureus (S. aureus)* (4.3%). Single pathogen was detected in 20 (14.2%) samples whereas combinations were found in 121 (85.8%) samples. *Bacillus cereus* and *E. coli* were the most frequently detected pathogens followed by the *S. Typhi*, *P. aeruginosa*, and *Staph. aureus*. The percentage occurrence of isolated pathogens was 31% in *B. cereus*, 31% in *E. coli*, 18% in *S. Typhi*, 5% in *P. aeruginosa*, and 3% in *Staph. aureus*.

Conclusion: *Pseudomonas aeruginosa* showed resistance against Amoxicillin and Cefotaxime, whereas *S. aureus* was found resistant against Cefotaxime. Statistical analysis using one way Analysis of Variance revealed that Ofloxacin and Gentamicin had significant ($p<0.05$) differences against all isolates as compared with other antibiotics used in this study.

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One billion people have poor access of water and approximately 2.6 billion lack of access to basic sanitation facilities worldwide.[1] Approximately 10-11 million children die before the age of 5 in low and middle income countries. Among many other infectious diseases, diarrhea is a major cause of morbidity and mortality in human. There has been much less progress in reducing diarrhea over the past decade, and 21% mortality rate was estimated in children of <5 years of age throughout the world.[2] Approximately 2 million children die in low and middle income countries due to diarrhea.[2] It accounts for 12% of all deaths due to infectious disease in the world. Approximately 90% of diarrhea cases in children are in low and middle income countries.[2] Among different types of diarrhea, acute watery diarrhea is more common with a morbidity rate of 80% and mortality rate of 50% in children.[3] Persistent diarrhea has a morbidity rate of 10%, but with an increased risk of death due to malnutrition. Globally, persistent diarrhea has a mortality rate of 35%, but Asia is proved to be more responsible for more than half of diarrheal deaths. Diarrhea can be caused by a variety of microorganisms that may be viral, bacterial, and protozoan. Most diarrhea causing microbes have a medium to low virulence.[4,5] There are 2 pathophysiological mechanisms by which bacteria causing disease. Either they produce toxins that affect the intestinal fluid, or by the intrusion of the microbes. Cholera toxin causes the fluid secretion.[6] Transmission route of the bacteria is by the fecal-oral route by means of unhygienic water, food, person to person interaction, or by the direct contact. Diarrhea is transmitted by domestic storage containers.[7] Protection against diarrheal agents also relies on the microflora of the gastro-intestinal tract. Most of the pathogens are killed by the hydrochloric acid. People with low level of gastric acid are more prone to contact diarrhea than others. Interrelated factors such as family formation, sanitizing conditions, availability of clean water and food, household environment, society trends, policies, and individual behavior also affect the rate of morbidity and mortality by diarrhea.[8] Antibiotics are not generally recommended to children with infectious causes.[9] But, antibiotics are given in case of cholera and dysentery. Zinc is used to reduce the severity and duration of the disease and lower the incidence of diarrhea in the following 2-3 months.[10] In 1979, oral rehydration therapy (ORT) was introduced and became an important task for the Control of Diarrhoeal Disease (CDD) program.[10] Oral rehydration therapy contains sodium, a carbohydrate, and water. Keeping in view the importance of the disease and associated losses, the present study was conducted with the objective to know the occurrence of the bacterial pathogens responsible for diarrhea in Faisalabad District, Punjab, Pakistan and to engender information regarding the effectiveness of commonly used antibiotic against diarrhea.

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Methods. This cross-sectional study was designed on patients admitted in gastroenteric wards or units of hospitals with the history and signs of diarrhea, dysentery, and dehydration between April and July 2014. A total of 141 stool samples by gender and from different age groups were collected during the mentioned time period from District Headquarter Hospital, Faisalabad, Pakistan.

Regarding the inclusion and exclusion criteria, patients with the signs and symptoms of diarrhea, dysentery, and dehydration were included and those without these signs were excluded. Samples were obtained in sterile containers, properly labelled and transported to the Cary-Blair transport medium (Oxoid, UK) along with the complete data sheet and under aseptic conditions to the Department of Microbiology, Postgraduate Research Laboratory, Government College University, Faisalabad, Pakistan.[11]

For the isolation and purification of bacterial pathogens, stool samples were inoculated first on the Nutrient agar (Scharlau, Spain) plates by swabbing, and incubated at 37°C for 24 hours. For purification, the different colonies from initial growth were streaked on MacConkey's agar (Oxoid, UK) and *Salmonella shigella* agar (Oxoid, UK) plates, and incubated at 37°C for 24 hours.[12]

Identification was carried out on the basis of cultural characteristics on different media and microscopic examination of stained smears through Gram staining procedure. Biochemical characterization was carried out using catalase, methyl red, indole production, Voges Proskauer, coagulase, oxidase, and triple iron sugar tests.[13-15] For antibiotic susceptibility testing, the agar disc diffusion method (Kirby-Bauer method) was performed using the Muller-Hinton agar medium (Oxoid, UK). The antibacterial susceptibility of bacterial isolates was determined against commonly selected used antibiotics including Ofloxacin, Gentamicin, Amoxicillin, and Cefotaxime. Turbidity of inoculum was adjusted to 0.5 McFarland and diameter of zone of inhibition were measured in mm.[16,17] Minimum inhibitory

concentration (MIC) is the lowest concentration of the antibiotics, which prevent visible growth of microbes. It was measured by Micro-broth dilution method using 96-well microtitration plate. Muller-Hinton broth (Oxoid, UK) was used with phenol red (0.01%) as an indicator.[18] The data was statistically analyzed by computing coefficient of variance and comparing the mean with standard deviation and one-way Analysis of Variance (ANOVA) using the Minitab.[15]

Results. A total of 141 specimen of diarrheal stool were collected. One hundred pathogen response rate was found and different bacteriological agents responsible for diarrhea from the same specimen were isolated. The patients were categorized into 3 age groups. First group (G1) aged from 1 day to 5 years (n=60), second group (G2) aged between 5 and 30 years (n=45), and third group (G 3) aged 30 years onwards (n=36). The 57 (40.4%) samples were male patients and 84 (59.6%) were females.

Single pathogen was detected in 20 (14.2%) samples whereas combinations were found in 121 (85.8%) samples. *Bacillus cereus* (*B. cereus*) and *Escherichia coli (E. coli)* were the most frequently detected pathogens followed by the *Salmonella typhi (S. Typhi)*, *Pseudomonas aeruginosa (P. aeruginosa)*, and *Staphylococcus aureus (S. aureus)*. The percentage occurrence of isolated pathogens was 31% in *B. cereus*, 31% in *E. coli*, 18% in *Salmonella typhi*, 5% in *P. aeruginosa*, and 3% in *S. aureus.* The growth of *E. coli* was observed as pink, shiny, smooth, round colonies with raised surface and varying growth pattern on MacConkey's agar. *Bacillus cereus* has a large, smooth, pink colonies with mousy smell on MacConkey's agar. Lactose non-fermenter colonies on the MacConkey's agar and central black, small size colonies with smooth to rough in appearance on the Salmonella-Shigella agar were identified as *Salmonella spp*. *Pseudomonas aeruginosa* was found to be non lactose fermenter with colorless, irregular, and round colonies with sweet odor on the MacConkey's agar. Smooth, creamy, and yellow colonies on the Staphylococcus 110 agar were observed for *S. aureus* (**Figures 1A-1D**). Gram's staining was carried out to identify the bacterial isolates on the basis of their morphology, arrangement, and staining characters. Different isolates showed the difference in these parameters that helped in their identification (**Figure 2**). Different biochemical tests were used to confirm the bacterial isolates including methyl red, Voges Proskauer, catalase, indole production, coagulase and oxidase tests. The results are shown in **Table 1**.

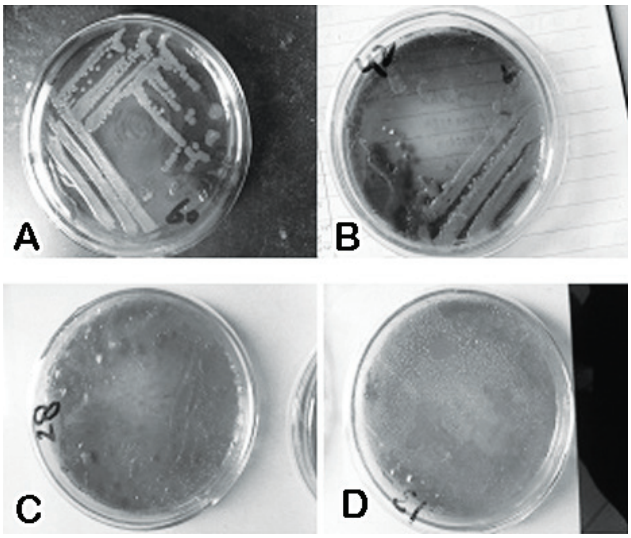

Figure 1 - Culture characteristics of bacterial isolates on different media. **A)** *Escherichia coli* on MacConkey's agar, **B)** *Bacillus cereus* on MacConkey's agar, **C)** *Salmonella typhi* on *Salmonella shigella* agar, and **D)** *Pseudomonas aeruginosa* on *Salmonella shigella* agar.

Table 1 - Biochemical test results of isolated bacterial pathogens.

Biochemical test	*S. aureus*	*B. cereus*	*E. coli*	*S. typhi*	*P. aeruginosa*
Catalase	+	+	+	+	+
Methyl Red	-	+	+	+	-
Vogues proskauer	+	+	-	-	-
Coagulase	+	NA	-	NA	-
Indole production	-	-	+	-	-
Oxidase	-	-	-	NA	+
Glucose	+, A	+, A	+, A	+, A	+, A
Sucrose	+, A	+, A	+	-	NA
Lactose	-	-	+, A	-	-

(+) Positive; (-) Negative, NA - not applicable, A - acid formation only, *S. aureus - Staphylococcus aureus, B. cereus - Bacillus Cereus, E. coli - Escherichia coli, S. typhi - Salmonella typhi, P. aeruginosa - Pseudomonas aeruginosa*

The diameter of zones of inhibition was measured in mm. *Bacillus cereus* showed the highest sensitivity against Ofloxacin with a mean diameter of 33.447±SD and least sensitivity against Cefotaxime as 12.617±SD. *Escherichia coli* (revealed highest sensitivity against Gentamicin with 13.448±SD mean diameter, and it was found resistant against Cefotaxime. *Salmonella typhi* exhibited the highest sensitivity against Gentamicin with a diameter 13.448±SD whereas found resistant against Cefotaxime. *Pseudomonas aeruginosa* displayed highest sensitivity against Gentamicin with 15.583±SD mean diameter and indicated resistance against Cefotaxime and Amoxicillin. *Staphylococcus aureus* presented highest sensitivity against Ofloxacin with the

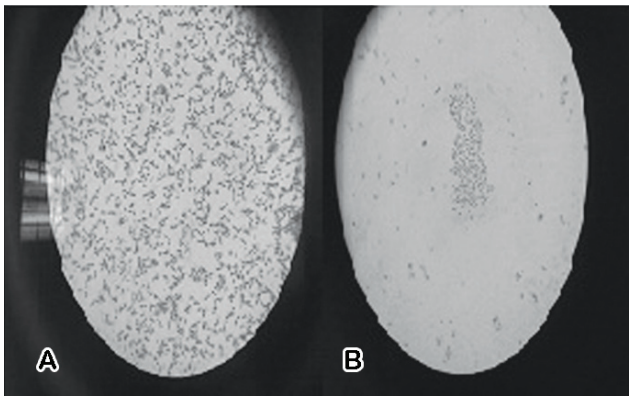

Figure 2 - Microscopic view of bacterial isolates (100x) A) Gram positive rods and B) Gram negative rods.

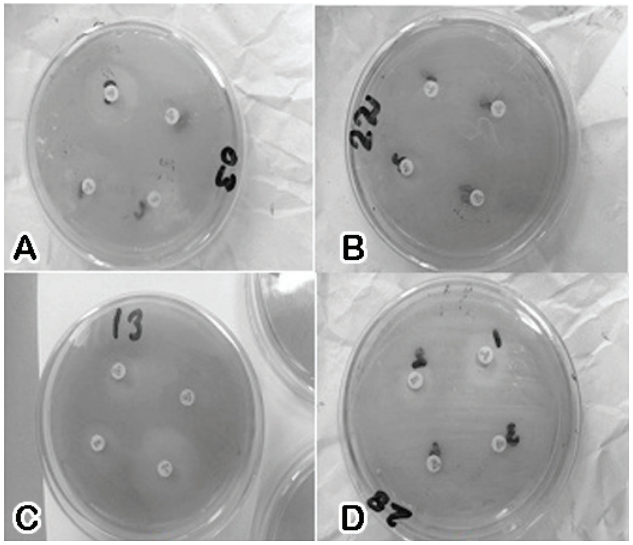

Figure 3 - Zones of inhibition shown by different antibiotics against bacterial A) *Bacillus cereus*, B) *Escherichia coli*, C) *Salmonella typhi*, and D) *Pseudomonas aeruginosa.*

28.00±SD mean diameter and found resistant against Cefotaxime (**Table 2** & **Figures 3A-3D**). The MIC of different antibiotics was measured as the minimum concentration of the antibiotic that inhibited the visible growth of microorganisms. Statistically, *B. cereus* was highly sensitive against Metronidazole with the mean ± SD of 7.526±2.513 and *E. coli* against Ciprofloxacin with mean ± SD of 2.491±0.770. *Salmonella typhi* was highly sensitive against Amoxicillin with a mean ± SD of 2.362±0.792 whereas *P. aeruginosa* showed highest sensitivity against Ofloxacin with a mean ± SD of 7.916±2.574. *Staphylococcus aureus* was highly sensitive against Ciprofloxacin with a mean ± SD of 2.340±0.854 (**Table 3**).

Discussion. The present study was performed to determine the type and occurrence of bacterial pathogens involved in diarrheal cases from patients of both gender and different age groups from District Faisalabad, Punjab, Pakistan. The antibacterial susceptibility pattern of isolated bacteria was also studied against commonly used antibiotics. From the collected stool samples, bacterial pathogens such as *B. cereus*, *E. coli*, *Salmonella*, *S. aureus,* and *P. aeruginosa* species were isolated and identified. Previous studies[19-21] also reported the presence of similar type of bacteria in the stool specimens of diarrheal patients. Vibrio cholera and *Shigella spp*. were reported to cause the most severe form of diarrhea namely, dysentery. In the present study, both were isolated, but their prevalence was non-significant as compared with *B. cereus*, *E. coli*, *Salmonella spp*., *S. aureus,* and *P. aeruginosa*. Moreover, in the present study, the type of bacterial pathogen isolated from diarrheal patients did not show any correlation with the age group. Similarly, it was reported in previous study as well.[21]

Isolates were identified on the basis of colony characteristics on different selective media. Bacillus cereus showed pink color colonies on the MacConkey's agar due to the lactose fermentation. It forms large colonies because of adapting a wide range of environmental conditions.[21,22] *Salmonella typhi* produced small black color colonies on *Salmonella Shigella* agar as it produces the hydrogen sulphide. *Pseudomonas aeruginosa* did not ferment lactose, so it appeared colorless on the MacConkey's agar. *Staphylococcus aureus* showed yellow color colonies on the blood agar media. Isolates were identified by the separation of the lactose fermenter and non-lactose fermenter bacteria using the novel approach presented by Khan et al.[23] Isolates were confirmed on the basis of the results of biochemical tests. *Bacillus cereus* was confirmed in 93 samples as Gram positive rods with Methyl Red-Voges Proskauer (MR-VP) positive. Because of the accumulation of acids, pH of the medium drops as MR changes from yellow to red color and a tinge of red color in VP medium due to the production of non-acidic products.[14,15] *Escherichia coli* was identified in 67 samples with MR positive and VP negative as the addition of acids causing the pH of the medium falls, which MR altered from yellow to red color, and a shade of red color in VP medium due to the production of non-acidic products. The indole production test was also positive as *E. coli* has the capability to generate indole from tryptophan by using an enzyme tryptophanase and was identified in the presence of Kovac's reagent.[24] *Salmonella typhi* was confirmed in 39 samples as the indole negative and

Table 2 - Diameter of zones of inhibition of different antibiotics against bacterial isolates.

Bacterial isolates	Zones of inhibition in mm (Mean ± SD)			
	Ofloxacin	Gentamicin	Amoxicillin	Cefotaxime
Bacillus cereus (n=93)	33.447 ± 3.429	22.500 ± 3.110	23.277 ± 3.059	12.617 ± 2.889
Escherichia coli (n=67)	11.701 ± 2.082	13.448 ± 2.025	3.382 ± 1.985	0.886 ± 0.179
Salmonella typhi (n=39)	25.00 ± 6.130	14.692 ± 4.786	7.30 ± 3.72	0.1601 ± 0.0256
Pseudomonas aeruginosa (n=12)	15.583 ± 3.260	15.167 ± 2.552	0.00 ± 0.00	0.00 ± 0.00
Staphylococcus aureus (n=6)	28.00 ± 3.22	12.333 ± 2.160	5.83 ± 4.71	0.00 ± 0.00

Table 3 - Minimum inhibitory concentration (MIC) of different antibiotics against bacterial isolates.

Bacterial isolates	MIC of different antibiotics (Mean ± SD)			
	Ofloxacin	Ciprofloxacin	Amoxicillin	Metronidazole
Bacillus cereus (n=93)	0.078 ± 0.0064	1.821 ± 0.980	0.0478 ± 0.038	7.526 ± 2.513
Escherichia coli (n=67)	0.093 ± 0.077	2.491 ± 0.770	2.180 ± 0.770	0.124 ± 0.038
Salmonella typhi (n=39)	0.052 ± 0.040	2.240 ± 0.783	2.362 ± 0.792	0.268 ± 0.071
Pseudomonas aeruginosa (n=12)	7.916 ± 2.574	2.210 ± 0.803	0.00 ± 0.00	0.247 ± 0.080
Staphylococcus aureus (n=6)	0.052 ± 0.040	2.340 ± 0.854	0.064 ± 0.050	0.234 ± 0.085

smooth, small, and colorless colonies due to lack of hydrogen sulfide (H_2S) production on the *Salmonella Shigella* agar. *Pseudomonas aeruginosa* was identified in the 12 stool samples due to the oxidase positive test indicating the presence of cytochrome enzyme. *Staphylococcus aureus* was confirmed in 6 samples showing catalase, and coagulase test positive due to the presence of catalase enzyme that produce bubbles on reacting with the hydrogen peroxide (H_2O_2) and releasing the products as water (H_2O) and molecular oxygen (O_2).[14]

The percentage occurrence of *B. cereus* was the highest (66%) among all the isolates. Al-Khatib et al[25] also reported similar results. Second most frequent isolated pathogen in all age groups was *E. coli* (48.5%). The reason for this may be the improper sanitization of utensils and unhygienic conditions in this region of the world. Third most frequently isolated pathogen was the *Salmonella* (27.7%), which was prevalent in G3 group and less common in G1 and G2. Frequency of occurrence of different bacterial pathogens in this work was almost similar to the work of Manikandan and Amsath with approximately 10% variations.

In this study, commercially available and commonly used 4 antibiotics such as Ofloxacin, Gentamicin, Amoxicillin, and Cefotaxime (Sigma, Saint. Louis, MO, USA) were selected for antibacterial susceptibility testing. Ofloxacin and Gentamicin are broad-spectrum antibiotics that inhibit the protein synthesis whereas Amoxicillin and Cefotaxime inhibit peptidoglycan of the both Gram positive and Gram negative cells. The overall results of disc diffusion assay revealed that all isolated pathogens showed statistically significant (p<0.05) sensitivity against Ofloxacin and Gentamicin whereas they were resistant to Amoxicillin and Cefotaxime.

The *B. cereus* exhibited partial resistance against Amoxicillin and Cefotaxime, whereas it was sensitive to Gentamicin and Ofloxacin. The result coincides with the work of Kiyomizu et al.[26] *Escherichia coli* showed resistance against Amoxicillin and Cefotaxime and sensitivity against Gentamicin and Ofloxacin. *Salmonella* presented sensitivity against Ofloxacin and resistance against Amoxicillin. A study conducted in the Bangladesh[27] also revealed similar results. *Staphylococcus aureus* showed sensitivity against Ofloxacin and resistance against Cefotaxime. *Pseudomonas aeruginosa* exhibited sensitivity against Ofloxacin and Gentamicin, whereas it was resistant against Amoxicillin and Cefotaxime.

In conclusion, *B. cereus* was the most frequently isolated organism from stool samples of diarrheal patients of both gender, and all age groups followed by *E. coli, S. typhi, P. aeruginosa,* and *S. aureus*. The overall percentage of occurrence of diarrheal cases was higher in children and females compare with males. Ofloxacin and Ciprofloxacin were found to be the most effective antibiotics for the treatment of diarrhea as compared with the Amoxicillin and Metronidazole used in this study. As such there is no limitation of the present study; however, further studies need to be conducted in other districts of Punjab Pakistan. It is hoped that these findings will be helpful to establish an effective treatment and may help control diarrhea.

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