

Original Research

Prevalence of Antibiotic-Resistant Airborne Bacteria along Roadsides in Rahim Yar Khan, Pakistan

Nusrat Naz^{1*}, Faiz ul Hassan Nasim^{1**}, Tariq Sultan Pasha²

¹Chemistry Department, The Islamia University of Bahawalpur, Bahawalpur, – 63100, Pakistan

²Department of Occupational Health, Faculty of Public Health and Health Informatics, Umm Al Qura University, Makkah, Kingdom of Saudi Arabia

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Abstract

The prevalence of antibiotic-resistant airborne bacteria was examined at seven different localities along the urban roads of Rahim Yar Khan. Airborne bacteria from the respiratory zone were sampled three times a day and five times a year using gravity deposition on nutrient agar plates. Six antibiotics – ampicillin, penicillin, streptomycin, clarithromycin, ciprofloxacin, and ceftriaxone – were used to screen antibiotic-resistant airborne bacteria. In this study, antibiotic-resistant airborne bacteria were detected at all sampling sites, with the highest antibiotic resistance observed in a residential area (RA). The airborne bacteria showed maximum resistance to streptomycin. The airborne bacteria of winter season were more resistant (43%) to tested antibiotics than airborne bacteria of any other season. These results specify that the pollutant exposure risk factor is different at each sampling site because of the potential contribution of various point sources. These findings of the study will be helpful to public health professionals and policy makers to develop effective interventions to combat adverse health impacts of bio-aerosols on the local population.

Keywords: antibiotic resistance, airborne bacteria, bioaerosols, Rahim Yar Khan

Introduction

Bioaerosols constitute about 50% of atmospheric aerosols. The organic particles in the bioaerosols may be bacteria, fungi, viruses, toxins, endotoxins, pollens, metabolites, or any other biological materials [1]. Over the last few decades, bioaerosol research has improved

significantly because of its adverse impact on human health. These biological origin particles are suspended in the air. In external ambient >30% of all aerosols (>0.2 μm in sizes) are of biological origin. Bioaerosols disperse for considerably long distances. However, their infectivity depends upon their ability to survive in the environment and their exposure to a vulnerable host. Survival or viability of bioaerosols pertains to their ability to reproduce (replicate), whereas infectivity of bioaerosols is its ability to initiate infection. The viability and infectivity of bioaerosols

*e-mail: nusratnaz.epa@gmail.com

**e-mail: faiznasim@hotmail.com

is contingent upon multiple factors such as ambient humidity, temperature, oxygen, other pollutants, and radiation [2]. The health effects associated with bioaerosol exposure include infectious diseases, respiratory diseases, skin diseases, allergies, cancer, and damage to the immune system [3]. Various types of area sources and point sources are continuously contributing to airborne bacterial load. The major forms of area sources are dry soil, plants, and animals, whereas the point sources include dead and decaying animal bodies, sewage sludge disposal sites, waste treatment plants, and solid waste disposal sites [4]. It is reported that on the agricultural sites the airborne bacterial load was maximum in summer and during crops harvesting time [5]. The risk of airborne pathogen transmission was greater in the close vicinity of waste disposal sites and it goes on decreasing as the distance increases. It has been reported in a study that workers were at little risk of airborne bacterial infections at a distance of 10 km from a sewage sludge disposal site and there was no risk of airborne viral infection at such a distance [6].

In urban areas, airborne bacterial concentration varies greatly at different sites due to different point sources and vehicular turbulence. The vehicular intensity adds to dust suspension with the subsequent addition of bioaerosols. Meteorological parameters like wind direction, wind speed, temperature, and relative humidity determine the suspension, transportation, and deposition of airborne microbes [7]. A study on the infectious disease Q fever revealed that wind direction plays a major role in transporting and transmitting the Q fever bacterium *Coxiella burnetii* from cattle farms to human communities. This bacterium multiplies in sheep placenta and when deposited on soil is transported by wind. Humans may get infected either by inhalation or by animal contact. In humans, the incubation period of bacterium is 20 days with subsequent signs of Q fever [8]. Hospitals provide a

potentially favourable environment for the proliferation and transport of antibiotic-resistant airborne microbes. The indoor environment of hospitals accumulates infectious bioaerosols, which by air transport lead to nosocomial infections in outdoor environments. The common antibiotic-resistant airborne bacteria in hospital environment include *Staphylococcus aureus*, *staphylococcus epidermidis*, *E. coli*, *Bacillus* sp. *Proteus mirabilis*, and *Streptococcus* sp. [9]. In a study at different sites of the University of Karachi, it was noted that airborne bacteria isolated from the Centre of Molecular Genetics laboratories were more resistant to antibiotics due to continuous use of antibiotics in the laboratories [10].

Different motives subsidizing to increase the resistance of microbes are frequent use of antibiotics for treatment of infectious diseases, the use of pesticides for crops, the use of antimicrobial lotions and soaps, dishwashers, and antibiotics for food production and for animal treatment. Antibiotic resistance ability develops in steps, starting from less susceptible strains to fully resistant microbial strains as observed in the case of penicillin-resistant *Pneumococci* sp. [11].

A study of antibiotic-resistant airborne bacteria from few labs or indoor environments of a few buildings or from a few hospitals is not enough when the concern is interlinked to public health. Yet the mode of genetic transfer from bacterial strains of different environments is not elucidated. In such a situation, the study of environmental microbes becomes essential to approximate the enormity of emerging antibiotic resistance. The aim of the present study was to estimate the presence of antibiotic-resistant airborne bacteria in outdoor environment of urban areas. Entire airborne bacterial sampling was carried out in a respiratory zone by passive sampling/gravity deposition to understand the real health risk posed by interaction of human beings and antibiotic-resistant airborne bacteria.

Table 1. Seasonal variation of airborne bacterial population on nutrient agar plate at different sampling sites.

Sampling Sites	Bacterial count (cfu/plate)				
	Summer	Rainy	Autumn	Winter	Spring
IA	298	220	259	224	269
RS	254	125	121	148	261
MA	199	107	175	94	166
RA	230	135	231	181	218
BS	156	102	114	107	229
CN	224	115	109	226	105
VA	116	175	204	201	145
Total count in season	1477	979	1213	1181	1393

Table 2. Seasonal variation of antibiotic-resistant airborne bacterial population at different sampling sites.

Sampling Sites	Antibiotic Resistant bacteria (cfu/plate)	Total count at each site (cfu)				
	Summer	Rainy	Autumn	Winter	Spring	
IA	Nil	Nil	49	19	02	70
RS	Nil	Nil	08	248	Nil	256
MA	Nil	51	21	12	05	89
RA	65	55	10	185	17	332
BS	14	14	27	Nil	108	163
CN	18	08	06	38	04	74
VA	Nil	189	05	Nil	Nil	194
Resistance Percentage	07 %	32 %	10 %	43%	10 %	---

Materials and Methods

Study Sites and Sampling Frequency

Rahim Yar Khan District, located in the southern part of Punjab Province in Pakistan, is backward in terms of human development indicators [12]. The linkage between poverty and antimicrobial resistance is an established fact. In developing countries, where an estimated 78% world's population lives, 23% of deaths are attributed to infectious and parasitic diseases [13]. Antibiotic-resistant infections cause considerable and avoidable costs to the public health system [14].

To assess the magnitude of antibiotic-resistant airborne bacteria, seven sampling sites covering an area of about 20 km were selected along a main road of the city. These sampling sites, because of human socio-economic activities, were marked as industrial area (IA), railway station (RS), market area (MA), residential area (RA), bus stop (BS), CNG pumps area (CN), and the last sampling site was the vegetative area (VA). The airborne bacterial sampling was done in triplicate three times in a day: morning, noon, and evening (M, 7-8 am, N, 1-2 pm, E, 6-7 pm) on three consecutive days. The airborne bacterial sampling was carried out five times in a year to monitor the diversity of antibiotic-resistant airborne bacteria in different seasons: summer (May-June), rainy (August-September), autumn (November), winter (December-January), and spring (March-April).

Sampling of Airborne Bacteria

Airborne bacterial sampling was done by gravity settle plate method. Sterilized petri plates were loaded with Nutrient Agar as reported by [15] and transported to sampling sites in sterilized conditions. The petri plates were exposed to ambient air in the respiratory zone of 1.5 m above ground level. After 15 minutes of exposure, petri plates were closed, sealed, and

transported to a laboratory for incubation at 37°C. After 24 hrs incubation, the total cfu/petri plate was counted and the whole plate was scraped with the help of a sterilized rubber spatula and transferred to eppendorf to make glycerol stocks. These glycerol stocks were stored at -70°C for further analysis.

Screening of Antibiotic-Resistant Airborne Bacteria

Six antibiotics – penicillin (s), ampicillin (s), streptomycin (s), ciprofloxacin (inj.), ceftriaxone sodium (inj.), and clarithromycin (inj.) – were selected for screening of antibiotic-resistant airborne bacteria. The minimum concentration of each antibiotic used for screening was 10 µg/ml of nutrient agar. For preparing a fresh bacterial sample, 10 µl of each preserved bacterial sample was inoculated to 3 ml of nutrient broth and incubated overnight on a shaker at 37°C.

Using nutrient broth, a suitable dilution was prepared from fresh bacterial culture to get the countable number of colonies on a petri plate. The autoclaved nutrient agar was cooled to about 60-40°C and then the selected antibiotic was added in it as per required concentration. The nutrient agar flask was swirled to homogenize the antibiotic with agar. The nutrient agar-containing antibiotic was poured in petri plates and allowed to solidify.

Only 50 µl of diluted bacterial culture was used for spreading on each petri plate. For control, the spreading of bacterial culture was done on a nutrient agar petri plate without any antibiotic. For each antibiotic screening the bacterial culture spreading was done in triplicate. About 30 min were given for absorption of bacterial culture on agar surface. The petri plates were incubated overnight at 37°C in an incubator. The number of colonies formed on each petri plate was counted and reported as cfu/plate along with standard deviation.

At each sampling site, results were elucidated by calculating the percentage of antibiotic-resistant airborne bacteria.

Results

Airborne bacterial samples were screened individually in triplicate to estimate the level of antibiotic resistance. The average count of antibiotic-resistant bacteria from each individual sample was used to interpret the level of antibiotic resistance at different sampling sites and in different seasons (Table 2).

Antibiotic Resistance and Airborne Bacteria

Results indicated the presence of antibiotic-resistant airborne bacteria at all sampling sites and in all seasons. However, a prominent variation in the extent of antibiotic-resistant airborne bacteria was observed in the present study. This may be due to variations of the surrounding atmosphere and different human activities that contribute to bioaerosol suspension and transport in the air [16]. In the present study, six antibiotics were selected on the basis of its mechanism of action to bacterial cell. The bactericidal antibiotics penicillin and ampicillin block the cross linking of bacterial cell wall by inhibiting the transpeptidase enzyme. The streptomycin binds with 30s rRNA subunit and blocks the protein synthesis. Similarly, clarithromycin belongs to the class of macrolide compounds and binds to 50s rRNA subunit to impair with protein synthesis. Ciprofloxacin belongs to the 2nd generation of fluoroquinolone compounds and stops the DNA synthesis by inhibiting DNA gyrase enzyme. Ceftriaxone belongs to the 3rd generation of cephalosporin compounds and acts as a bactericide by inhibiting the transpeptidase enzyme in order to stop the bacterial cell wall synthesis [17]. Among the tested antibiotics, airborne bacteria showed maximum resistance to streptomycin, followed by penicillin. On the other hand, airborne bacteria were least resistant to ceftriaxone and ciprofloxacin as indicated by Fig. 1. Ciprofloxacin-resistant airborne bacteria were noted only in the rainy season. The results

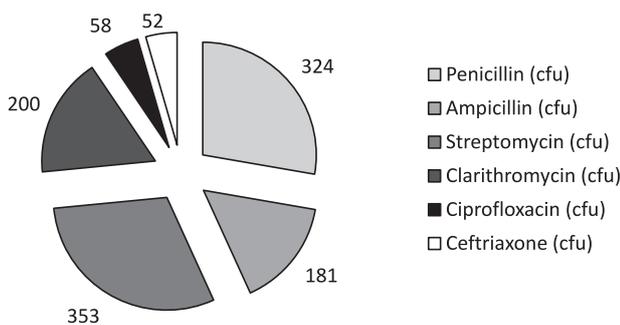


Fig. 1. Airborne bacteria and level of resistance to different antibiotics.

illustrated that cephalosporin and fluoroquinolone compounds in the form of ceftriaxone and ciprofloxacin, respectively, are more potent antibiotics than any other antibiotics. The magnitude of antibiotic resistance exhibited by airborne bacteria was:

$$\text{streptomycin} > \text{penicillin} > \text{clarithromycin} > \text{ampicillin} > \text{ciprofloxacin} > \text{ceftriaxone}$$

It is evident that due to prolonged use of old antibiotics, airborne bacteria have developed maximum susceptibility to streptomycin, penicillin, clarithromycin, and ampicillin. Whereas the 2nd and 3rd generation antibiotics in the form of ciprofloxacin and ceftriaxone respectively are strong antibiotics and airborne bacteria are least resistant to these antibiotics.

Variation of Antibiotic Resistance at Different Sampling Sites

The highest level of antibiotic-resistant airborne bacteria was noted at RA with a total of 332 bacterial cfu resistant to different antibiotics. The second sampling site with an increased health risk was RS, where antibiotic resistance level was 256 cfu during the monitoring of airborne bacteria. The lowest count of antibiotic-resistant airborne bacteria was noted at IA and CN, with a total of 70 and 74 resistant airborne bacterial cfu, respectively. A more interesting situation was observed at VA, where antibiotic resistance was observed in rainy and autumn seasons only. In summer, winter, and spring seasons, airborne bacteria at VA were not resistant to any tested antibiotics. Similarly, at RS antibiotic-resistant airborne bacteria were present only in autumn and winter seasons. At RA, antibiotic-resistant airborne bacteria were prevailing throughout the sampling period. The intensity of antibiotic resistance at different sampling sites was:

$$\text{RA} > \text{RS} > \text{VA} > \text{BS} > \text{MA} > \text{CN} > \text{IA}$$

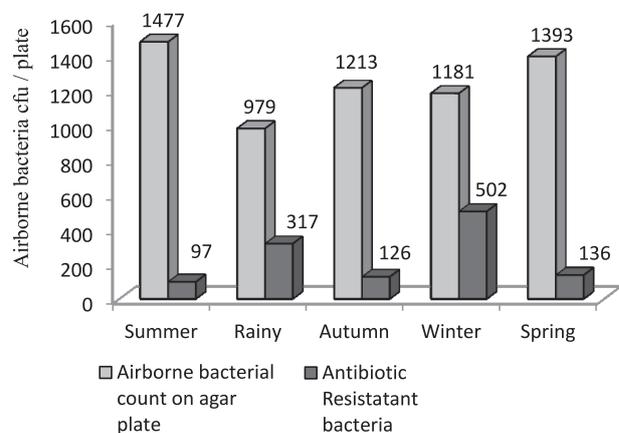


Fig. 2. Comparison of airborne bacterial load and antibiotic resistant bacteria.

Alteration of Antibiotic-Resistant Airborne Bacteria in Different Seasons

In comparing results of antibiotic-resistant airborne bacteria in different seasons, total bacterial population in different seasons (Table 1) was used to calculate the percentage of antibiotic resistance. The order of total airborne bacterial population in different seasons was:

summer > spring > autumn > winter > rainy

...whereas the succession of antibiotic-resistant airborne bacteria in different seasons was:

winter > rainy > autumn = spring > summer

It was interesting to note that although the highest bacterial load was observed in the summer, only 7% of total bacterial population was resistant to tested antibiotics in summer (Fig. 2). Contrary to this observation, in the rainy season we observed that airborne bacterial load was the lowest, but it showed a remarkable antibiotic resistance relative to other seasons. The highest percentage (43%) of antibiotic-resistant airborne bacteria was noted in winter.

Discussion

The results of a study on airborne biological threats indicated that gradual dispersion of pathogenic airborne microbes or any microbial product in the form of toxins or endotoxins can cause a serious health threat to distant community. This study also indicated that the public is generally unaware of these incidences, and infection may develop even several days after pathogenic exposure. The results implied that without regular surveillance, it is not possible to maintain public awareness of airborne microbial threats. For the purpose to protect the community from serious health risks associated with airborne bacterial outbreak, early detection, warning, and response direction are necessarily required [18].

The main focus of our present research was to monitor the presence of biological threat agents in the form of antibiotic-resistant airborne bacteria and to assess the risk of public exposure to this menace. Our results indicated the presence of antibiotic-resistant airborne bacteria at all sampling sites. We noted that among the six tested antibiotics, airborne bacteria have developed maximum vulnerability to penicillin, streptomycin clarithromycin, and ampicillin due to its persistent usage. On the other hand, the airborne bacteria were least susceptible to ciprofloxacin and ceftriaxone, which are 2nd and 3rd generation antibiotics. The results may be supported by the findings wherein airborne bacteria sampled from a dairy farm and its surroundings, showed the highest resistance to ampicillin, nalidixic acid, trimethoprim, and tetracycline among 14 tested compounds [19]. Human pathogens like *Escherichia coli*, *Shigella* spp., *Salmonella* spp., and

Staphylococcus aureus have shown increasing trends toward commonly prescribed antibiotics like ampicillin, amoxicillin, penicillin, and tetracycline [20]. Other factors, like presence of metal, cadmium, or lead ions in the atmosphere also contribute to the development of antibiotic-resistant bacterial strains disseminated in air, causing an adverse health impact on our community [21]. It is alarming that the development rate of antimicrobial agents is slow compared to the emergence of resistance toward existing drugs. It is essential to formulate the strategy to fight the menace of drug resistance with iron hands [22].

The study of airborne microbes in an open environment is more complicated and difficult to interpret due to the interaction of different potential sources plus various anthropogenic and meteorological parameters. In the present study, depending upon human activities and anthropogenic factors, the magnitude of antibiotic-resistant airborne bacteria varies significantly in outdoor environments of all sampling sites. As far as the sources of antibiotic-resistant airborne bacteria are concerned, it is reported that its major portion is contributed by human beings, plants, animals, and soil sources. Transmission of airborne bacteria may be by biotic and abiotic modes like water, the food chain, and direct contact and through air – posing a serious health threat. A study from Greece has reported the presence of methicillin-resistant *Staphylococcus aureus* from salted and smoked ready-to-eat fish products [23]. Bacteria isolated from food animals also contain significant amount of antibiotic-resistant *E.Coli* [24]. Healthy pet animals (dogs and cats) serve as reservoirs of multidrug-resistant *staphylococci*, which may be transmitted to humans by animal interaction [25]. Many physical processes that are not limited to gravitational force and include wind speed, wind direction, diffusion and evaporation govern the dispersion of the bioaerosols produced from infectious persons by coughing and sneezing activities [26]. It is still to be answered regarding which proportion of infection will be transmitted by aerosolized particles, what proportion of airborne infectious agents are viable, and what proportion of inhaled infectious agent will cause disease? Currently, accurate airflow visualization techniques are employed to illustrate the transmission of airborne infectious particles to preclude its health effects [27].

A vast diversity of bacterial Phylotypes is reported to be present on the human body and contribute to the surrounding environment [28]. A study from India has reported a higher concentration of bioaerosols (bacteria and fungi) at large human gathering sites during a fair as compared to the non-fair period. The study also portrayed the higher proportion of antibiotic-resistant airborne bacteria during and after the fair [29]. The appearance of antibiotic-resistant bacteria in the air is due to excessive use of antibiotics to control infection, person-to-person contact emissions, hospitalization, overcrowding, and treatment of respiratory ailments.

In the present study, the highest magnitude of antibiotic-resistant airborne bacteria was noted at RA compared to other sampling sites, signifying human beings as one of the potential sources of antibiotic-resistant airborne bacteria. The result may be supported by findings of a study, carried out at Metro Station in Shanghai, China, that crowded and closed environments may accelerate the transmission of antibiotic-resistant strains of *Staphylococcus* [30]. This is further supported by a U.S. research study showing that environmental contamination of hospital rooms by methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococci* from prior room occupants increases the acquisition chances of these microbes for new patients [31]. Similarly, the presence of erythromycin- and tetracycline-resistant genes have been reported in all bioaerosol samples collected from hospital rooms in Canada [32]. The methicillin-resistant airborne *staphylococci* were found to be prevalent at heavy traffic stations in Istanbul, implying an increasing trend of community-acquired infections [33]. Stefano Bassetti and his coworkers, from North Carolina has reported that virus-induction increases the nasal transmission of *Staphylococcus aureus* in air from healthy volunteers [34]. The airborne *Staphylococcus aureus* strains from residential homes are more resistant to antibiotics than from homes in the outer environment [35]. Another research study indicated that genes responsible for antibiotic resistance are distinct for environmental and human-associated microbes. Antibiotic resistance ability is reported to be a function of microbial ecological constraint [36].

The frequent use of antibiotics to control infections promotes human pathogens, each having specific micro-environment. If the ecosystem of a specific micro-environment is altered by the release of a large amount of antimicrobials, the dynamic of atmosphere will be altered with the selection of resistant genes, having unpredictable consequences on human health [37]. The most common reported antibiotic-resistant airborne bacteria include vancomycin-resistant *Enterococci*, methicillin-resistant *Staphylococcus aureus*, penicillin-resistant *Pneumococci*, and multidrug-resistant *mycobacterium tuberculosis* [38].

The second potential source of antibiotic-resistant airborne bacteria is generated from animal feeding houses. The use of antimicrobial products for livestock and agriculture in order to enhance production has increased the extent of antibiotic-resistant airborne pathogens related to human significance [39]. In livestock, the selection of antibiotic-resistant genes occurs in gastro-intestinal bacteria that are excreted in manure. The ultimate disposal of animal excreta is land application with transport and dispersion of antibiotics and antibiotic-resistant determinants to the natural environment. The antibiotic-resistant biological cell may survive for a few weeks or a few months, depending on species and meteorological parameters, but antibiotic-resistant genetic elements can persist regardless of

cell survival [40]. On the dispersion mode of airborne bacteria at swine feeding operations in Ohio, Christopher F. Greena conducted a comprehensive study and reported that a maximum airborne bacterial load was observed inside the animal feeding confinement with progressive dispersion in the upwind direction. It was also noted that 76% of airborne bacterial population in the vicinity of a swine feeding operation was *staphylococcus aureus* [41]. The presence of tetracycline-resistant genes was more abundant in bioaerosol samples collected from animal confinements as compared to bioaerosol samples collected from human-occupied buildings [42, 43].

Seasonal shifts greatly influence the survival of airborne microbes. In the present study, the prominent variation of antibiotic-resistant airborne bacteria was noted in different seasons. In a similar study, in order to estimate biological diversity, bioaerosol samples were collected from various points of Greater Mexico City (GMC) for winter, spring, summer, and autumn seasons in 2008, 2010, 2011, and 2012. The 16SrRNA gene sequencing indicated the presence of *Enterobacteriaceae*, *Bacillus* sp., *Pseudomonas* sp., *Acinetobacter* sp., *Erwinia* sp., *Gluconacetobacter* sp., *Proteus* sp., *Exiguobacterium* sp., and *Staphylococcus* sp from all samples of low atmosphere at GMC [44]. The airborne microbes associated with suspended particulate matter of size PM_{10} and $PM_{2.5}$ were found to differ from each other in different seasons. The seasonal change indicated great variability of microbial community with plant-associated bacteria dominating in summer and spore-forming bacteria in winter [45]. In our research study, the magnitude of antibiotic-resistant airborne bacteria was maximum in winter, followed by the rainy season. Although studies to assess seasonal variability of the microbial community are well reported, to the best of our knowledge there is no report regarding the variation of antibiotic-resistant airborne bacteria throughout the year. Only a few researchers have tried to estimate antibiotic-resistant determinant from air samples of two extreme seasons, i.e., summer and winter. In one such study, the seasonal dynamics of biotic contaminants was estimated in swine confinement buildings during summer and winter. The results indicated great variability of bacterial abundance and microbial community related to seasonal shifts. The biotic contaminant samples were further utilized for quantifying six tetracycline-resistant genes by real-time PCR. Out of six tested genes, the frequency of four tetracycline-resistant genes was higher in winter samples and it was significantly correlated with microclimatic factors like air speed, PM_{10} , and total suspended particles [46]. The genetic study indicated that in the case of multidrug-resistant *Staphylococcus* strains the most phenotypic resistance was related to cell wall permeability and efflux pump, thereby reducing the risk of genetic transfer [47].

The World Health Organization has conducted a survey to assess the magnitude of globally emerging antibiotic resistance. The report has pointed out the gaps

of resistance surveillance, lack of standards in sampling and analysis techniques, shortage of data availability and data sharing, and a coordination gap among all stakeholders [48]. It is suggested that antibiotic resistance may not be due to the consistent use of life-saving drugs, but instead other factors like the use of disinfectants, antibacterials, and antimicrobials. The current need is to understand microbe's natural ecology and interaction of potential community risk components before the emergence of a serious health threat.

Conclusion

Surveillance of antibiotic resistance is a major tool to assess the health risk of drug-resistant airborne microbes. In the present study, antibiotic-resistant airborne bacteria were detected at all sampling sites, with supreme antibiotic resistance observed at RA. The airborne bacteria showed maximum resistance to streptomycin. The airborne bacteria of winter were more resistant to tested antibiotics than airborne bacteria of any other season. There are several ways to control the spread of antibiotic-resistant airborne bacteria, such as reducing the use of antibiotics, prohibiting over-the-counter (OTC) sale of antibiotics in pharmacies, using sub-clinical antibiotics dosages, using the antibiotics as per physician prescription, and – most important – by improving hygienic conditions. Research also plays an important role in overcoming antibiotic resistance by understanding bacterial flora, the mechanism of antibiotic resistance development, and disseminating antibiotic-resistant airborne bacteria. There is a need for developing broad-spectrum antibiotics in order to depose most of the outdoor antibiotic-resistant bacteria. Moreover, public awareness for the rational use of antibiotics and national health plans for stringent control of infectious diseases cannot be neglected.

Future Prospects

Research is ongoing for the identification and molecular characterization of antibiotic-resistant airborne bacteria. It will help to assess what types of antibiotic-resistant airborne bacteria are prevailing in the studied atmosphere and what may be its health impacts.

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Conflict of Interest

The authors declare no conflict of interest.

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