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Antibiotic Resistance Profiling of Pseudomonas Species Isolated from Cloacal Swab of Domestic Pigeons

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ABSTRACT: *Antibiotics are used to treat a number of bacterial infections. However, overuse or misuse of antibiotics has raised serious concerns against antibiotic resistance amongst bacteria. Hence, antibiotics are becoming inefficient in treating bacterial infections leading to an increase in mortality rate worldwide. The domestic animals especially birds are a major source of transmission of antibiotic resistant bacteria in human through excrement and cause bacterial diseases in human. The aim of the present study was to assess the efficacy of different antibiotics prior to their prescription as a measure to prevent antibiotic resistance in bacteria. For this 120 cloacal swab samples were collected from the domestic pigeons of District Narowal to isolate Pseudomonas sp. and assess the efficacy of different antibiotics prior to their prescription as a measure to prevent antibiotic resistance in bacteria. Antibacterial activities were evaluated by performing antibiotic susceptibility pattern of Pseudomonas isolates against 18 commercially available antibiotic discs [Trimethoprime (TMP), Clarithromycin (CLR), Gentamicin (GEN), Chloramphenicol (C), Ampicillin (AM), Streptomycin (S), Kanamycin (K), Nitrofurantoin (F), Amoxicillin (AX), Tazobactam (TPZ), Imipenem (IPM), Meropenem (MEM), Levofloxacin (LEV), Nalidixic acid (NA), Ceftriaxone (CRO), Amikacin (AK), Tetracycline (TE) and Ciprofloxacin (CIP)] by using Kirby-Bauer disc diffusion method. Amongst these antibiotics, notably Pseudomonas sp. showed highest sensitivity to Clarithromycin (93.94%), Ampicillin (100%), Amikacin (93%) and Nalidixic (100%). This study established a general antibiotic resistance pattern of commercially used different antibiotics for commonly encountered clinical isolates. Moreover, antibiotics susceptibility tests (AST) should be carried out prior to prescribing antibiotics to the patient. Additionally, the antibacterial activities of local clinical isolates and change in bacteriological profile due to indiscriminate use of antibiotics associated with appearance of multiple drug resistant strains should be evaluated. It was concluded that preventive measure and their implementation is quite necessary to control antibiotic resistance and domestic pigeons can be a carrier of Pseudomonas species and can transmit through their fecal material to humans and other animals.*

Keywords: *Antibiotic resistance, pseudomonas, Antibacterial susceptibility, cloacal swab*

INTRODUCTION

Antibiotics are the foremost antibacterial chemicals for curing bacterial infections and are broadly used in the treatment and elimination of bacterial diseases (Anupurba et al., 2003). They perform their activity by destroying or inhibiting the growth of bacteria. Antibiotics are not only effective in fighting against microbial infections but also effective in controlling the bacterial infections (Baddour et al., 2006). However, overuse and misuse of antibiotics, poor hygiene and contamination control mechanisms had led to antibiotic resistance in bacteria (Shaikh, 2017). Nowadays, infectious pathogens in wild life have become increasingly important throughout the world, as they have substantial impacts on human health and agricultural production (Kabir, 2016). Transmission of various bacterial pathogen from free living birds to humans have been reported such as *E. coli, Pseudomonas aeruginosa*, *Klebsiella* spp., *Salmonella* spp., *Pasteurella* spp., *S. aureus* and *Proteus* spp. were recovered from hoopoe, ibis, sparrow, doves and quails with variable rates (Effat and Moursi, 2005; Hedawy and El-Shorbagy, 2007).

Various species of free living birds, because of their propensity to nest and roost near human activity, may harbor and disseminate various species of bacterial microorganisms to domestic birds and animals (Abdallah and Khalil, 2016). Undomesticated pigeons are classified under the species *Columba livia* and belonging to the Columbidae family, and order Columbiformes. The present population of this species found in most cities are originated from caged and domesticated pigeons at least five thousand years ago in their native territory (Vasconcelos et al., 2018). Public initiated to take care for these birds and gave them food and provided a favorable environment for these birds to adapt. Currently, undomesticated pigeons are found in large numbers at public locations, and often in close contact with man (Gasparini et al., 2017).

Pseudomonas aeruginosa is the most prevalent bacterial species. It can be highly pathogenic causing 50-100% morbidity in experimentally inoculated 4 week old chicklings (Pike et al., 2017). *Pseudomonas aeruginosa* is a Gram negative, medium sized (0.5- 1.0 x 1.5-5.0µm) bacteria that grows on common culture media usually producing a water soluble green pigment chemically made-up of fluorescein and pyocyanin and a specific plummy odor can often be recognized (Degaim et al., 2019). *Pseudomonas aeruginosa* is toxins producing pathogen which causes infections in stages: bacterial adherence, colonization, invasion and dissemination and systemic of toxemic disease (Prince et al., 1991).

Minimum 60 various types of human pathogens have been already isolated from birds. However, research performed with undomesticated pigeons aiming to determine their zoonotic

potential in the prevalence of infections to humans are limited to the isolation of microorganisms, not considering pathological aspects of the infection in these birds (Benskin et al., 2009). In spite of being occurred and frequently isolated, the reports of conveyance of such pathogens to humans from pigeons are very rare, usually occurring with immunosuppressed persons (Haag-Wackernagel et al., 2004).

On the basis of these facts, the aim of the study was to fill knowledge gaps about the prevalence of the pathogen and related virulence factor in pigeon populations. To determine the extent to which pigeons might harbor pathogens and pose a risk to the human population in Pakistan, the pigeons around Narowal were screened for the presence of Pseudomonas as pathogens to public health. Therefore, the study aimed to describe naturally occurring infection of *Pseudomonas aeruginosa* and other species of pseudomonas in domestic pigeons (*Columba livia domestica*) from Narowal, Pakistan and to assess their antibiotic resistance patterns against different antibiotics.

MATERIALS AND METHODS

A cross sectional study was conducted at Microbiology Department Sughra Shafi Medical Complex, Narowal, Punjab Pakistan. A total of 120 samples of domestic pigeons of both gender (Cloacal swabs) were collected from different areas of District Narowal, during a time span of one year. Data was analysed for mean and standard deviation by using SPSS (Statistical Package for Social Sciences) version 24.0.

Sample collection and processing

Each sample was collected in a sterile container. The sample container was labeled with the details of source, date and time of collection and transported to laboratory for analysis within one hour of collection.

Isolation and Identification of *Pseudomonas*

After sample collection, samples were inoculated in Asparagine broth was used as enrichment media for *Pseudomonas* (Silva et al., 2011). Positive result was indicated by green color of broth. Positive samples were again inoculated on asparagine agar plates for the isolation and purification purpose. Firstly incubated plates were incubated for twenty-four hours at 37ºC. After incubation, isolated colonies were further purified using streak plate method.

Antibiotic Susceptibility Testing (AST)

Kirby diffusion disc technique was performed to find out the antibiotic susceptibility pattern of isolated *Pseudomonas*. Following antibiotics were evaluated: Trimethoprime (TMP), Clarithromycin (CLR), Gentamicin (GEN), Chloramphenicol (C), Ampicillin (AM), Streptomycin (S), Nitrofurantoin (F), Amoxicillin (AX), Imipenem (IPM), Meropenem (MEM), Levofloxacin (LEV), Ciprofloxacin (CIP), Ceftriaxone (CRO), Amikacin (AK), Kanamycin (K), Ampicillin (AM), Tetracycline (TE), Nalidixic acid (NA), Tazobactam (TPZ)

Results

Results were interpreted as resistant (R), sensitive (S) and intermediate (I). The antibacterial susceptibility pattern of different antibiotics was summarized in table 1. The antibacterial susceptibility pattern showed that 6.06% (n=2) *Pseudomonas* species were resistant while 93.94% (n=31) were sensitive towards Trimathoprim (TMP) and Clarithromycin (CLR) respectively. The antibacterial activity of antibiotic Gentamicin (CN) against *Pseudomonas* sp. showed antibacterial susceptibility pattern as intermediate 27.27% (n=9), resistance (18.18% n=6) and sensitivity of 54.55% (n=18). It was noticed that antibacterial activity of antibiotic Chloramphenicol (C) against *Pseudomonas* was 18.18% (n=6), 60.61% (n=20) showed intermediate growth and 21.21% (n=7) showed sensitivity. The antibacterial susceptibility pattern of Ampicilin (AM) against *Pseudomonas* revealed 100% (n=33) resistant. The antibacterial susceptibility pattern of Streptomycin (S) had shown that 36.36% (n=12) intermediate growth, 57.58% (n=19) resistance and 6.06% (n=2) shown sensitivity against *Pseudomonas*. The antibacterial activity of antibiotic Nitrofurantoin (F) against *Pseudomonas* was 81.82% (n=27) with resistance and 18.18% (n=6) showed sensitivity whereas no intermediate growth was recorded.

The antibacterial activity of antibiotic Amoxicilin (AX) against *Pseudomonas* showed 100% (n=33) resistance and no intermediate and sensitivity growth was recorded. The antibacterial activity of antibiotic Tazobactam (TPZ) against 36.36% (n=12) were with intermediate growth, 57.58% (n=19) were showed resistance and 6.06% (n=2) showed sensitivity. The antibacterial susceptibility pattern of Imipenem (IPM) had shown that 18.18% (n=6) *Pseudomonas* species showed intermediate growth, 33.33% (n=11) showed resistance and 48.48% (n=16) showed sensitivity. The antibacterial activity of antibiotic Meropenem (MEM) of *Pseudomonas* showed 54.55% (n=18) intermediate growth, 33.33% (n=11) showed resistance and 12.12% (n=4) showed sensitivity. The antibacterial activity of antibiotic Levofloxacin (LEV) against *Pseudomonas* 42.42% (n=14) showed intermediate growth 33.33% (n=11) showed resistance and 24.24% (n=8) showed sensitivity. The antibacterial activity of antibiotic Ciprofloxacin (CIP) against *Pseudomonas* sp. 8.06% (n=2) were showing intermediate 51.52% (n=17) were showing resistance and 42.42% (n=14) were showing sensitivity. The antibacterial activity of antibiotic Ceftriaxone (CRO) against *Pseudomonas* is of *Pseudomonas* 6.06% (n=2) were showing intermediate 93.94% (n=31) were showing resistance and whereas *Pseudomonas* does not shown any sensitivity. The antibacterial activity of antibiotic Amikacin (AK) against *Pseudomonas* sp. was 100.00% (n=33) were showing sensitivity and whereas

Pseudomonas sp. did not show any Resistant and intermediate growth. The antibacterial activity of antibiotic Kanamycin (K) against *Pseudomonas* was 9.09% (n=3) were showing intermediate 36.36% (n=12) were showing resistance and 54.55% (n=18) were showing sensitivity. The antibacterial activity of antibiotic Tetracyclin (TE) against *Pseudomonas* was 6.06% (n=2) were showing intermediate 51.52% (n=17) were showing resistance and 42.42% (n=14) were showing sensitivity. The antibacterial activity of antibiotic Nalidixic Acid (NA) against *Pseudomonas* 93.94% (n=31) were showing resistance and 5.05% (n=2) were showing sensitivity. Whereas, *Pseudomonas* sp. did not show any intermediate growth.

Fig. 1 showed the sensitivity of *pseudomonas* sp. against different tested antibiotics. Amikacin (AK), Trimethoprime (TMP) and Clarithromycin (CLR) were noticed as most effective against concerned bacteria whereas Fig. 2 showed the resistance pattern of *pseudomonas* species against tested antibiotics. Results revealed that majority of tested antibiotic have very low antibiotic efficacy against isolates.

Table 1. Antibiotic resistance profiling of *Pseudomonas* species isolated from cloacal swab of domestic pigeons

Antibiotic	Sensitive	Intermediate	Resistant
Trimethoprime (TMP)	93.94%	ND	6.06%
Clarithromycin (CLR)	93.94%	ND	6.06%
Gentamicin (GEN)	54.55%	27.27%	18.18%
Chloramphenicol (C)	21.21%	18.18%	60.61%
Ampicillin (AM)	ND	ND	100%
Streptomycin (S)	6.06%	36.36%	57.58%
Nitrofurantion (F)	18.18%	ND	81.82%
Amoxicillin (AX)	ND	ND	100%
Tazobactam (TPZ)	6.06%	36.36%	57.58%
Imipenem (IPM)	48.48%	18.18%	33.33%
Meropenem (MEM)	12.12%	54.55%	33.33%
Levofloxacin (LEV)	24.24%	42.42%	33.33%
Ciprofloxacin (CIP)	42.42%	8.06%	51.58%
Ceftriaxone (CRO)	ND	6.06%	93.94%
Amikacin (AK)	100%	ND	ND
Kanamycin (K)	54.55%	9.09%	36.36%
Tetracyclin (TE)	42.42%	6.06%	51.52%
Nalidix Acid (NA)	5.05%	ND	93.94%

Abbreviation: Not Detected (ND)

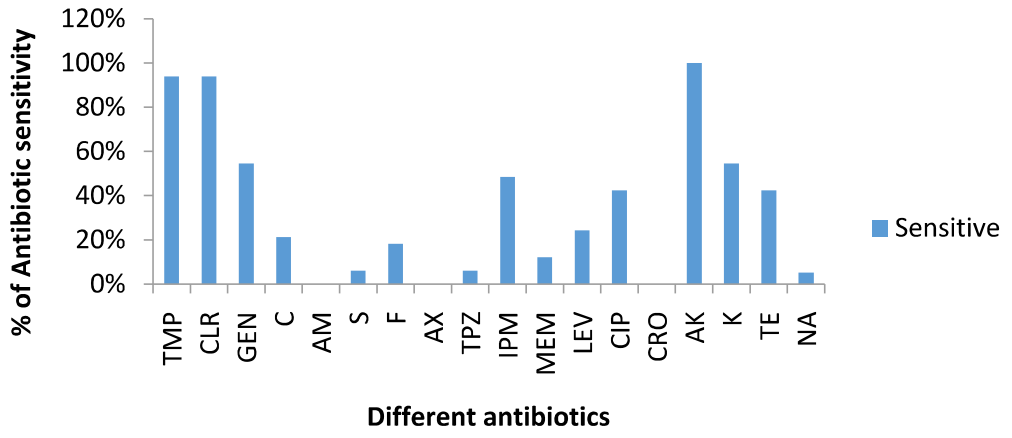

Fig. 1. Comparative sensitivity of different antibiotics against *Pseudomonas*

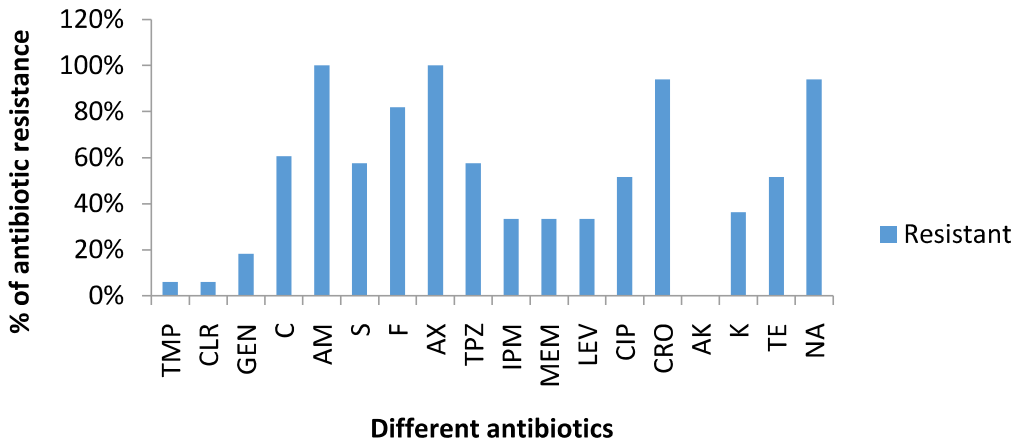

Fig. 2. Comparative Resistance of Pseudomonas against antibiotics

DISCUSSION

In this study, 120 cloacal swab samples were collected from the domestic pigeons of District Narowal. Clinical isolates of *Pseudomonas* sp. were obtained from 33 (27.5%) samples which were identified through conventional culture and biochemical tests. *Pseudomonas* species were the most common bacteria and is found in 7.8% of related species of birds. Aerobic bacteria were reported in the microbiota of the large intestine of many animals as well as humans (Lister et al., 2009). *Pseudomonas* species were also found in birds and under favorable conditions they developed a symbiotic relationship. It may be associated with many infectious conditions e.g. rhinitis, sinusitis, laryngitis, septicemia and hemorrhagic enteritis (Bailey et al., 2009).

This study aimed to test various antibiotics for antibacterial activity against isolated *Pseudomonas species*. Primarily, the purpose was to compare the antibacterial activities of these antibiotics against *Pseudomonas* isolates.

To the best of our knowledge, studies on AMR profiles of these microorganisms in related birds in companionship are not available. There is possibility to transmit infections and resistant traits to other species and also to humans, making it a concern for public health that needs attention. In a previous study, *Pseudomonas aeruginosa* was found in 7% of the birds of prey with blood infections (Vidal et al., 2017) and oral lesions while in our study, the frequency was 25%, even in healthy birds. Mostly, the bacterial strains showed resistance to all the antibiotics while only one showed resistance to gentamicin. In contrast, the above study reported 100% resistance to clindamycin and 21% to gentamicin.

Before selecting an antibiotic for any bacterial infection, the information for antibiotic susceptibility test (AST) should be collected (Arayne et al., 2002). Accurate performance of susceptibility tests were very important to obtain good results in accordance with the condition and treatment (Ryan, 1970). These symbiotic and opportunistic bacteria were critical and its important to have identification of new antibiotics to cope with MDR properties (Pragasam, 2018). AST should be applied before prescribing the antibiotics to patients. Furthermore implementation of preventive measures was recommended for the control and prevention of antibacterial resistance. This study helped in establishing a general antibiotic resistance pattern of currently in use different antibiotics for commonly encountered clinical infections caused by *Pseudomonas sp.* A study is recommended in future to evaluate the antibacterial activities of local clinical isolates and also subsequently identify change in bacteriological profile due to indiscriminate usage of antibiotics associated with the appearance of multiple drug resistant strains.

CONCLUSION

On the basis of this study, it was concluded that domestic pigeons can be a carrier of *Pseudomonas* species and can transmit through their fecal material to humans and other animals. Preventive measures must be taken to avoid transmission. Moreover, Trimethoprime and Clarithromycin were best to use as both showed least resistances as compared to all other antibiotics.

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Development and Quality Evaluation of Nutritious and Healthy Biscuits for Dogs

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ABSTRACT: *Pet food awareness among people is increasing day by day due to humanization trends towards pets. The pet owners are searching for various quality pet foods having different tastes. Therefore, there is an increase demand of premium products i.e. organic, raw and natural pet foods which are giving lots of choices for pet owners. The present study entails the preparation of dog biscuits by using cheap and organic sources. Orange, apple and banana peels were used in the preparation of dog biscuits in order to utilize fruits wastes. Nutritional, heavy metals and aflatoxins analyses was done to evaluate useful aspects of biscuits for dogs. Aflatoxins were absent in biscuits and heavy metals were also within permissible levels showing harmlessness of biscuits for pet dog. The biscuits were offered to pet dogs for sensory evaluation and were found acceptable. The results from nutritional analysis showed that the prepared biscuits for dog are a nutritive food having enough high calories of 407.55 Kcal/100g in order to fulfill dietary requirements of dogs. It was concluded that prepared biscuits for dogs were nutritionally fit for dog's health.*

Keywords: *Nutritional analysis, Aflatoxins, Heavy metals, dogs, biscuits*

INTRODUCTION

Pet dogs are being treated everywhere in the world as "family members" now-a-days. Pet food industry is multimillion businesses as the commercial dog foods are too costly to provide to pet dogs by common people (Deuri et al., 2019). There are so many commercial dog foods available in market with respect to dog age, breed type and life stages. In Pakistan the trend of setting pet food industries is on the rise as people have now became aware.

Dog biscuit is taken as full diet supplement for pet dogs and other pet animals. Dog biscuits are likely as hard and dry entity. A proper diet and its nutritional aspects are very important for dog's good health. It is much needed that biscuits have proper amount of nutrients and other ingredients (Akinrinmade and

Akinrinde, 2011). Common dog biscuits typically contain between about 60-70% wheat flour (Scaglione and Gellman, 1988). Normally dog biscuits are composed of food grade cellulose, salt, best binder to hold the whole matrix and flavors and taste enhancers.

The dog biscuits are prepared by ingredients of low calories so that they can be consumed by a dog in large amount without creating obesity problem (Andersen and Harpe, 1990). Pet manufacturer have art to mix different blends in appropriate proportions of meat cuts, vegetables, cereals, fish, minerals and vitamins. The nutritionally balanced pet food is prepared by pet food manufactures so that pet can enjoy delicious balanced diet. There are so many choices for pet owners to prepare pet food of great quality by mixing raw materials for wet and dry formulations for pet treats.

Dog biscuits are ready to eat pet food product which are conveniently prepared and are inexpensive. The dietary principles and digestives requirements are kept in mind while selecting ingredients and preparing biscuits (Kulkarni, 1997). Wheat flour, sugar, salt, baking powder, milk and different flavors are main ingredients for biscuits making (Obasi et al., 2012). Peels powders play an important role in enhancing nutritional aspects. The apple peels help manage oxidative stress and maintain inflammation within the normal range. Banana peels are also useful from nutrition point of view as they contain 6-9% protein (dry matter) and 20-30% fiber. Similarly, orange peels are the source of vitamins A and C and contain antioxidants which are helpful in boosting immunity. These are useful for medicinal purpose as well e.g. in flu, colds and infections (Wolfe et al., 2003; Kalpna and Mital, 2011).

Different manufacturers of numerous pet foods are preparing complementary; dry snacks, treats or raw and wet foods for dogs (Beynen, 2020). There is lack of study regarding pet food and this study is the first step towards developing and evaluating dog food. This study was carried out with the objectives of formulating an edible dog-food in biscuits form using locally available ingredients in Pakistan at lower price level, which confirm the main nutritional requirements. It is designed as such that all nutritional and energy requirements are kept in mind and the use of peels of different fruits and meat in preparation of biscuits is worth mentioning aspect in biscuits composition in order to add different important nutrients.

MATERIALS AND METHODS

Fresh fruits (Orange, apple and banana) were purchased from local market of Lahore. The fruits were peeled off. The peels were taken and washed carefully with water to remove dust and other particles. All the peels were then dried with neat cloth. The dried peels were then kept in drying oven for 12 hours at 70 ºC. The dried peels were

grinded to fine powder. The list of ingredients have shown in Table 1.

Table 1: List of ingredients for the preparation of biscuits for dogs

Sr. No.	Name of Ingredients	Percentage (%)
1	All purpose flour	32
2	Wheat Flour	15
3	Baking powder	0.4
4	Butter	5
5	Sugar	20
6	Milk	7
7	Orange peel powder	2
8	Apple peel powder	2
9	Banana peel powder	2
10	Salt	1
11	Meat pieces	3
12	Vanilla Essence	0.2
13	Cocoa Powder	0.4
14	Egg	7.5
15	Oil	2.5

Eggs, milk and sugar were firstly well batted and then all the ingredients were well mixed to get fine dough. The dough was rolled out on a thick sheet and was cut with a cutter in different desired shapes. The unused dough was again pressed and same procedure of rolling was repeated and biscuits were cut in different shapes. The tray was covered greased by oil and covered with butter paper which again was greased by oil and biscuits were placed for baking in preheated oven for 10 minutes at 180°C till the biscuits became golden brown.

Evaluation of Physico-Chemical Parameters

1. pH Determination

5% solution of the biscuits was prepared using water and pH was checked by pH meter.

2. Nutritional Analysis

The nutritional composition of the dog biscuits was determined and all the experiments were carried out in the laboratories of Food Department, PCSIR Laboratories Complex Lahore, Pakistan. The dog biscuits were grinded to fine powder and packed in air-tight polythene bag and were labeled for nutritional analysis. The percentages of moisture content, crude fire, ash and crude protein in the dog biscuits were determined by using official methods (AOAC, 1990). 5g of biscuits in powder form were weighed and placed in hot air oven at 130°C to a constant or at equilibrium weight. After three hours, the difference in weight was recorded as the moisture content and percentage was calculated. 0.5g of the powdered biscuit sample was weighed and placed in a pre-weighed china

crucible and ignited in an ash furnace maintained at 550°C till the fine ash was obtained. The percentage ash was calculated. The nitrogen contents were determined by using micro-Kjeldahl method and then multiplied by 6.25 to estimate the crude protein contents in biscuit samples. Percentage of carbohydrate was also calculated by the difference as depicted (AOAC, 1990).

3. Determination of Heavy Metal Contamination

The solution was prepared by taking 1g ash. The sample in crucible was taken, ignited and then placed in furnace. The obtained ash was dissolved in distilled water by adding nitric acid then filtered to make volume 100 ml using distilled water. Different metals (Lead, Cadmium, Copper, Nickel, Cobalt, Zinc and Mercury) were then measured by Atomic Absorption Spectrophotometer alongwith their respective standards (Bhowmik et al., 2008). Arsenic was determined by using Inductively Coupled Plasma.

4. Aflatoxin Determination

Aflatoxins in biscuits were determined by using thin layer chromatographic technique (Nisa et al., 2012). Aflatoxins were analyzed (Begum, 1985) in prepared biscuits for dog. 50g of biscuits in powdered form were taken and 250ml chloroform was added and then shaken for 30minutes. Then 50ml of sample solution was taken and dried on water bath. Volume was prepared and spotting was done on TLC plate as in depicted method (Romer, 1976). The different concentrations of standard were compared with the sample extract for the aflatoxin determination (AOAC, 2005). Total aflatoxins (AFB1+AFB2+AFG1+AFG2) were determined by given formula (Zahra et al., 2020).

$$\text{Aflatoxin B1 } (\mu g/kg) = \frac{S \times Y \times V}{W \times Z}$$

RESULTS AND DISCUSSION

The prepared dog biscuits were analyzed for its physicochemical and nutritional aspects. Presence of heavy metals in biscuits may have venomous effects on human health. So, the determination of heavy metals in biscuits was necessary. Analysis of heavy metals includes lead, cadmium, copper, nickel, cobalt, zinc, arsenic and mercury. The results are given in Table 2**.**

Table 2: Results of Physico-Chemical tests of prepared dog biscuits

Sr. No.	Parameter	Values
1.	pH	6.78±0.05
2.	Lead	ND
3.	Cadmium	ND
4.	Copper	ND
5.	Nickel	ND
6.	Cobalt	ND
7.	Zinc	0.1 ±0.01 ppb
8.	Arsenic	0.2 ±0.01 ppb
9.	Mercury	ND

*ND = Not Detected

The nutritional analysis results showed that the prepared dog biscuits may be considered as a nutritive and potential food entity as having high calories of 407.55±0.1 Kcal/100g in order to fulfill the dietary requirements.

Table 3: Results of Nutritional Analysis of prepared dog biscuits

Sr. No.	Parameter	Values
1.	Moisture (%)	7.70 ± 0.05
2.	Ash (%)	0.95 ± 0.02
3.	Protein (%)	8.18 ± 0.05
4.	Fat (%)	8.67 ± 0.05
5.	Fiber (%)	0.30 ± 0.01
6.	Carbohydrates (%)	74.20 ± 0.1
7.	Energy (Kcal/ 100g)	407.55 ± 0.1

Aflatoxins are the fungal metabolites which are declared as carcinogenic species by International Agency of Cancer on Research. These may affect biscuits quality to greater extent and also human health may affect (Zahra et al., 2015). In this study, prepared biscuits were found safe for dog consumption as there was no contamination of aflatoxins. It was depicted by all parameters that dog biscuits are secure for dog's health (Table 4).

Table 4: Aflatoxin Analysis of Dog Food Biscuits

Sr. No.	Aflatoxins	Concentration (ppb)
1.	B1	Not Detected
2.	B2	Not Detected
3.	G1	Not Detected
4.	G2	Not Detected

From this study it was obtained that the prepared biscuits are novel and distinctively provide good results for dog consumption with good calories.

CONCLUSION

The day by day increasing reliance on dogs for sanctuary and companion is thought to be responsible for the remarkable growth of the pet food industry, with analogous amplifying of demand for dog food by pet owners and dog breeders. The present work is focused on preparation of dog biscuits with best nutritional values. The nutritional analysis showed its efficacy for dogs use. No harmful metal was detected in prepared biscuits for dogs. To the best of our acquaintance, information on label claims of nutritional adequacy by pet food industries in Pakistan has not yet been validated.

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Retinoic Acid: A Potential Risk Factor for Congenital Heart Disease

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ABSTRACT: *Retinoic acid (RA), a derivative of vitamin A, plays an essential role in human beings during growth and development. Intake of vitamin A in body is by either animal/plant diet or use of medication to treat skin problems and cancer. Adequate intake of retinoic acid is critical as high and low quantities of retinoid lead to developmental defect by acting through retinoid receptors present in human body, which actively modulate many signaling pathways and control embryonic development and differentiation. Excess intake of RA cause congenital heart diseases (CHD) by up and down regulation of genes associated with CHD. Therefore, intake of retinoic acid or other analogous drugs especially during pregnancy enhances the risk of developing CHD. Physician and pharmacist should provide proper counseling to female patients about the benefits and risks of drugs prescribed during pregnancy. Furthermore, advancement in therapies likes surgery, angioplasty and tissue engineering used for the treatment of CHD. This review discusses an association between retinoic acid and CHD, its prevention and treatment options available.*

Keywords: *Congenital heart defect, birth defects, retinoic acid, retinoid receptors,*

INTRODUCTION

Vitamin A (retinol) plays an essential role in human beings that supports to control the cellular differentiation of epithelial tissue. Vitamin A exists in two dietary forms; one is found in animal products and second in plants as carotenoids (Niwa, 2018). They are essential biomolecules for embryonic development maintenance of body homeostasis. Many processes like cellular and epithelial growth, immune response, apoptosis, some nuclear receptors mechanisms and functions of retinoic acid receptor and retinoid X receptor (RXR) are influenced by retinoids (Perl et al., 2019).

Throughout early embryonic development, all-trans retinoic acid (atRA), are actively involved in development of pancreas, formation of body axis, cardiogenesis, vision process, breathing functions and neurogenesis. Additional intake of vitamin A results in teratogenisis and chances of birth defects in developing embryo. Intake of vitamin A and retinoids in excess is dangerous for pregnancy as it causes different anomalies to fetuses' central nervous system, skulls, limbs, face and eyes (Perl and Waxman, 2019). Both high and low levels of retinoic acid (RA) is a predominant risk factor for congenital heart defects with spectrum of developmental defects (Pace et al., 2018). Over intake of RA in the form of medications could contribute towards the development of CHD mostly due to lack of awareness. Henceforth, in this review, we have discussed the importance of adequate intake of RA and its role in the development of CHD and possible treatment modalities for CHD.

1. Intake of Retinoic acid

Retinoids used for the treatment of skin diseases like acne, scars, sun damage, wrinkling, skin tone problems, anti-aging as well as breast and ovarian cancer. In US, four retinoids introduced to fulfill the dermatological problems out of which one is 13-cis-retinoic acid isotretinoin (Accutane) used to treat acne. Use of this medicine showed negative impact on pregnant women. In US, some medicines are strictly ban during pregnancy including accutane and tegison. Exposure to 13-cis-retinoic acid bases cause deformities in developing fetuses and central nervous system deformities. Vitamin A known for its wider role in skin nutrition as enhancer since 1940s stimulates the new cell growth in skin. Early researches focused on the consumption of vitamin A orally but it was associated with some side effects like liver damage, hair loss and softening of skull (Dmitrovsky and Spinella, 2017).

2. Cellular metabolism of Retinoic Acid

Retinoic acid derived from vitamin A has specific role in organogenesis and cell growth. When cells need retinoic acid, they up-take retinol and convert it into retinaldehyde and retinoic acid. Two important enzymes catalyze this reversible reaction. Retinol dehydrogenases (ROLDH) convert retinol into retinaldehyde and retinal dehydrogenases (RALDH) convert retinaldehyde into retinoic acid. Active metabolites of retinoic acid are, all-Trans-RA, 9-cis-RA, and 4-oxo-RA. Retinoic acid serves as a ligand in nucleus and brings transcriptional changes through two families of receptors i.e., retinoic acid receptors

and retinoid X receptors (RXR) as described in fig. 1 (Fadel et al., 2020).

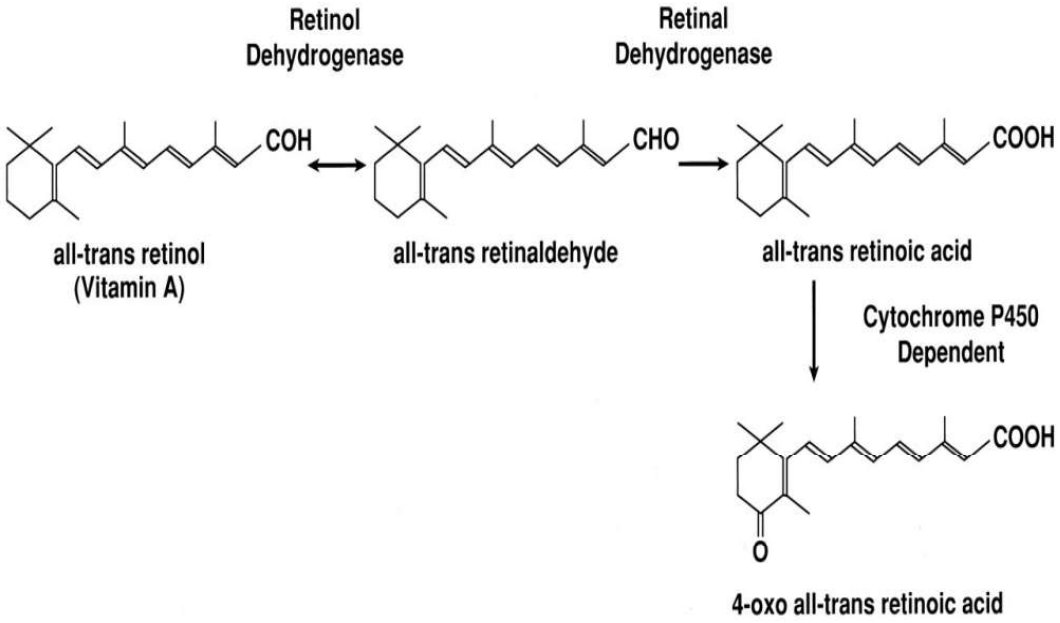

Fig. 1. Cellular Mechanism of Retinoic Acid (Fadel et al., 2020)

3. Retinoic acid as a risk factor of Congenital Heart Disease

Physiological concentrations of retinoic acids regulate the normal growth of embryo and cardio genesis (Wang et al., 2019). In vertebrate's development, congenital malformation occurs in many organs due to increased or decreased level of embryonic RA (Diaz et al., 2020). Some retinoid receptors are actively involved in many signaling pathways and regulate embryonic development & differentiation processes. Studies on animal models such as vitamin A deficient rat embryos resulted in heart developmental defects including aortic arch and ventricular outflow tract malformations (Wilson et al., 1949). Deficiency or excess of vitamin A mainly affects the valves or membranous septa of heart. It is thought that defect occur in development due to the up and down regulation of specific genes. Due to shortage of retinol certain genes repressed therefore, they not expressed properly. *Hox* gene is responsible for human embryonic development mainly in fourth week of pregnancy (Piersma et al., 2017). Altered function of this gene due to any mutation and loss of genetic control distorts the normal physiology. In humans, there is loss of CYP26A1 gene related with DiGeorge and Klipplel-Feil syndromes, which involves outflow tract defects (Pennimpede et al., 2010). Excess RA promotes the genetic expression of STRA6, LRAT, DHRS3, CRBP1, CRABP2, and CYP26A1, while it down regulate RBP4, RDH10 and ALDH1A2 expression (Amengual et al., 2014; Billings et al., 2013; Sandell et al., 2012).

This alters the specification of cardiovascular tissues during early development, left/right decisions and cardiac situs, anteroposterior patterning of the early heart, endocardial cushion formation, and the neural crest (Pan et al., 2007).

4. Treatments for Congenital Heart Disease

Many people get complete cure through surgery or medications. Most commonly prescribed medications are diuretics, beta-blockers, digitalis and anticoagulants.

Tet Spell: Tet Spell treated by using beta-blocker using Propranolol but in acute condition treatment with morphine or intranasal fentanyl and a vasopressor like phenylephrine or nor epinephrine are recommended to increase the vascular resistance. Oxygen plays a very efficient role in treating spells. It can help in movement of blood through lungs by decreasing the pressure of deoxygenated blood from the right to left ventricle with the help of VSD (Tsze et al., 2014).

Total Surgical Repair: Tetralogy of Fallot treated with surgery to improve the blood flow towards lungs to make sure the correct direction of oxygenated and deoxygenated blood. **Stem Cell Therapy:** is a potential treatment for difficult to treat disease. The basic aim is to stimulate the renewal of damaged tissues through stem cell therapy. Various injections depending on type of tissues and diseases recommended for this purpose. Different routes of injection administration like intracoronary, intravenous, and intramyocardial can be used for proper treatment (Brown et al., 2020).

Tissue Engineering: This technique is useful for the renewal of impaired tissue and is most effective in repair of damage or any organ like skeletal muscle, trachea and esophagus not present since birth (Londono et al., 2015). Tissue engineering reached to success in 1994 for the reproduction of tracheal cartilage. Some other techniques like bioengineering used to treat heart defects. If the stem cell embedded in extracellular matrix in the form of prosthetic graft and patches then it works more efficiently. Once the implantation is done in defective heart it alters or grow fast in physiological order (Ott et al., 2011).

Valve Replacement: Heart valves replaced with tissues or mechanical valve also offer an effective treatment of the damaged valves.

Balloon Angioplasty: Balloon angioplasty of pulmonary or aortic vein (BPV, BAV) is the best way for the treatment of congenital heart defects. Balloon angioplasty is a method of surgery in innate narrowing of aortic arch and re-narrowing of the aorta after an operation (Ammar, 2012). When there is narrowing in any part of heart's circulation system, balloon angioplasty is required which is also termed as balloon dilation. Balloon angioplasty helps in improving blood flow by opening the narrowed area of heart. Catheter, a thin

tube inserted at the top of leg into blood vessels. The tip of catheter inserted into the heart with a balloon, once the balloon reaches at the right place; it helps in opening the narrowed area. The balloon stretches the wall of artery and forces the narrow area to open. When balloon deflates, the catheters are taken out, whilst the injured part of leg is covered with bandage (Byrne et al., 2017).

CONCLUSION

Vitamin A (retinol) plays an essential role in human beings that supports to control the cellular differentiation of epithelial tissue. Intake of retinol occurs in the form of retinoids when administered to treat skin diseases like acne, scars, sun damage, wrinkling, skin tone problems, anti-aging as well as breast and ovarian cancer. Physiological concentrations of retinoic acids regulate the normal growth of embryo and cardiogenesis. Deficiency or excess of vitamin A mainly affects the valves or membranous septa of heart hence development of congenital heart defects. Retinoic acid is a non-genetic cause of CHD, which leads to the disturbance in any portion of heart, disturbed blood flow, blood flow in wrong direction or complete blockage. Intake of retinoic acid or other drugs during pregnancy enhances the risk of developing CHD. Hence, pregnant women should avoid taking any painkillers, drugs and alcohol. Good control of blood sugar, proper vaccination and complete regular checkup must be promoted. Some chronic condition like epilepsy requires the use of medications during pregnancy. Physician and pharmacist should provide proper counseling to female patients about the benefits and risks of drugs prescribed during pregnancy. Use of multivitamins and folic acid reduce the risk of birth defect in brain and heart (van der Bom et al., 2011). Food and Drug Administration has taken steps such as placing strict risk management protocols on the use of retinoids such as etretinate and isotretinoin, in order to prevent teratogenic defects (Abroms et al., 2006). Furthermore, advancement in therapies like surgery, angioplasty and tissue engineering have helped treat CHD. In depth understanding of underlying mechanism of role of RA in congenital heart disease in animal models and human subjects will help in reducing the risk factor.

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Awareness Regarding Hygienic Practices in Tuberculosis Patients of Sir Ganga Ram Hospital, Lahore

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ABSTRACT: *Worldwide, in a working-age population the tuberculosis poses a vital health care load among young adults. As the prevalence of tuberculosis increased, the rate of morbidity and mortality also increased. The objective of the study was to assess the awareness regarding hygienic practices among tuberculosis patients visiting Tertiary Care Hospital Lahore. A cross-sectional study was carried out at the chest department of Sir Ganga Ram Hospital, Lahore during November-2018 to January-2019.A sample of 100 samples of tuberculosis patients was selected through non-probability convenient sampling technique. Patients were assessed through pre-tested questionnaire. SPSS version 21.0 was used for data analysis. Among 100 participants, 55 patients were female and 45 patients were male. The prevalence of extra pulmonary TB is more in female 33% than male 21%. The mean age of the patients was 35 years. 63% TB patients cannot covered their face with mask when they cough or sneeze; 37 % TB patients had covered their faces with mask when they cough or sneeze. Total 79% TB patient's houses were good ventilated while 21% TB patient's houses or rooms were not ventilated. It was concluded that an increased family size, poor hygienic environment and low socioeconomic status were major determinants of tuberculosis.*

Keywords: *Mal-nutrition, tuberculosis, hygienic practices, Morbidity and Mortality rate*

INTRODUCTION

Tuberculosis is a contagious disease, primarily caused by bacteria specifically *Mycobacterium tuberculosis*, or *Mycobacterium africanum* (Fogel, 2015). The body's response to TB infection produces inflammation (Waitt and Squire, 2011) that may harm the lungs; the infection can be spread via blood from the lungs to other organs (Zak et al., 2016). TB is a communicable

disease involving impoverishment, mal-nutrition and poor immune system (Bhargava et al, 2019). Morbidity and mortality of TB are measured very high in developing countries (Pizzol et al., 2016). The illness is commonly diagnosed among economically disadvantage population (as in migrants, homeless people and children) or people lived in shut quarters (prisoners, and military forces) (Fares, 2011). TB is spread from inhalation of bacteria which is spread as droplets from the mucous secretion of infected persons by cough or sneeze (Glaziou et al., 2015). Symptoms of pulmonary tuberculosis square measure coughing that lasts three or more weeks; coughing up blood or bloody mucous secretion; temperature; night sweats; loss of body weight; pain in chest; chronic fatigue; and eating disorder (Lee, 2016). TB is an essential health problem that can be cured and is prevented. Approximately one-third of world's population is infected with TB; if left untreated, each infected person can infect 10 to15 people in one year (WHO report, 2015). Pakistan has been hierarchic fifth in twenty two high TB affected countries and fourth in countries wherever MDR-TB treatment has become very hard for health professionals (WHO report, 2013). Pharmacologically TB is treated with numerous drugs, particularly antibiotics. Under nutrition has conjointly been related to mal-absorption to key anti-TB medicine. Food insecurity and under nutrition inpatient with active TB will increase their risk of developing TB. Under nutrition will impair cell mediate immunity and increase the severity of TB illness (Ramachandran et al., 2013).

A study conducted by Maria A evaluated the patients' knowledge about tuberculosis (TB) and studied the effect of nutrition education on nutritional status of tuberculosis patients. The study was conducted at Gulab Devi Chest hospital Lahore. She did purposive sampling. Data was collected by filling questionnaire through interview Patients were counseled, given role of nutrition in managing disease. The patients were reexamined to evaluate the effect of 12 week intervention. Results of the study revealed improvement in BMI. The Hb and erythrocyte sedimentation rate (ESR) of patients also improved significantly after intervention ($p = 0.000$) and nutritional knowledge of the patients about tuberculosis improved significantly. The study showed that; nutrition education improved the knowledge about role of nutrition in TB and in turn nutritional status of the patients (Aslam et al., 2021).

Singh *et al*. (2018) conducted a research to study household environment effect on prevalence of tuberculosis and found that family members who were regularly (daily) exposed to smoke (second-hand smoke) inside the house were more prone to getting tuberculosis as compared with households where people do not smoke inside the house. Further, households having a finished wall less likely to get TB than the households with mud walls. There are multiple risk factors that are strongly associated with

Tuberculosis: smoke inside house, type of cooking fuel, separate kitchen, floor, roofing and wall material, number of persons sleeping in a room, sharing toilet and potable water with other households; and individual characteristics such as age, sex, educational attainment, marital status, place of residence and wealth index (Singh et al., 2018).

A case control study was conducted for one year in Guilan province of Iran to study the association between socio-environment and risk of pulmonary tuberculosis certain social determinants were found that influenced the host susceptibility to tuberculosis (TB) infections, and increase the risk of developing the disease. The average mean age of the TB cases was 51 years old. The majority of TB cases were from rural areas 71.3, while 28.7 were from urban areas. Significant differences ($P < 0.001$) were observed between the geographical conditions and distribution of the disease. A significantly inadequate UV irradiation was seen in the houses of the TB patients, compared to the control group ($P<0.001$). The hygiene of the houses seemed to be a significant risk factor ($P<0.001$) for TB infection. The results suggest that in the studied region several host and environmental factors were associated with higher risks of TB infection (Taher-Ghasemi, et al., 2016).

A study on TB patients in Hong Kong was carried out to examine sex differences in them and in the rate of difference in sign and symptoms. Data was obtained from chest service of TB in Hong Kong from the past five decades. The results of their study showed that the rate of TB during the past five decades was constantly greater in men than in women, regardless of age group, their results also showed that a higher proportion of women had extra-pulmonary tuberculosis than men; the main site of involvement was the lymph nodes (Tam et al., 2003).

Methodology

A prevalence based cross-sectional study was conducted at chest Department of Sir Ganga Ram Hospital, Lahore. Both genders between the age of 20-80 years were selected and the sample size was 100. Data were collected after the ethical approval from The University of Lahore by using the non-probability sampling technique. Non-cooperative and patients with other disorders were excluded. Pre-tested questionnaire/ Performa was used to collect data. Data were analyzed with the help of SPSS version 21.0. Frequencies and percentages were calculated to determine environmental conditions and hygienic practices.

RESULTS

Distribution of Gender and age cross tabulation

The results showed that majority of patients were females; 55% as compared to males; 45%. The minimum age of patients was 18 and maximum age was 70 and the mean age of patient's was ±34.64 year as shown in fig. 1.

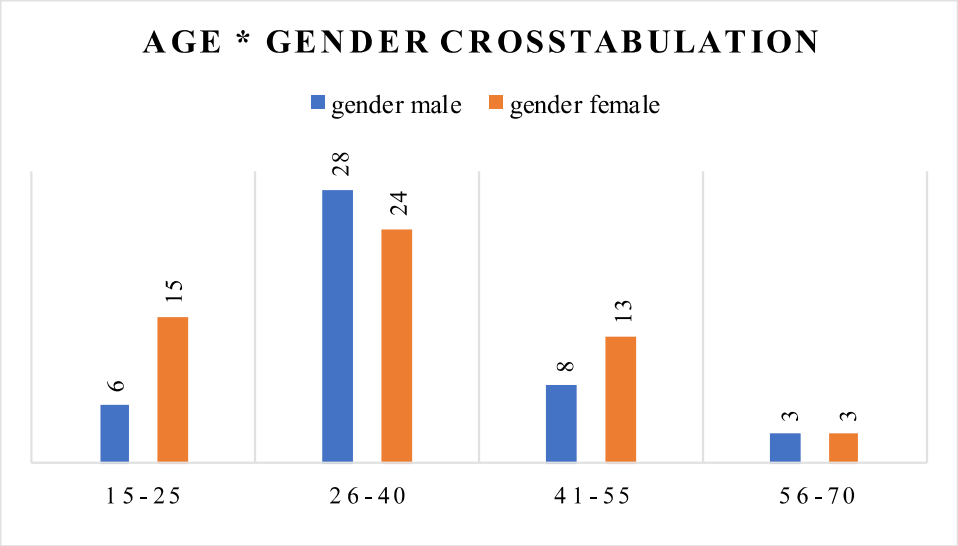

Fig. 1. Frequency distribution of Gender and age cross tabulation

Distribution of residence and family type cross tabulation

Results showed that Fifty-nine percent TB patients were from urban area 41% TB patients were from rural area and 60% TB patients lived in a joint family; while 40% TB patients lived in single family as shown in fig. 2.

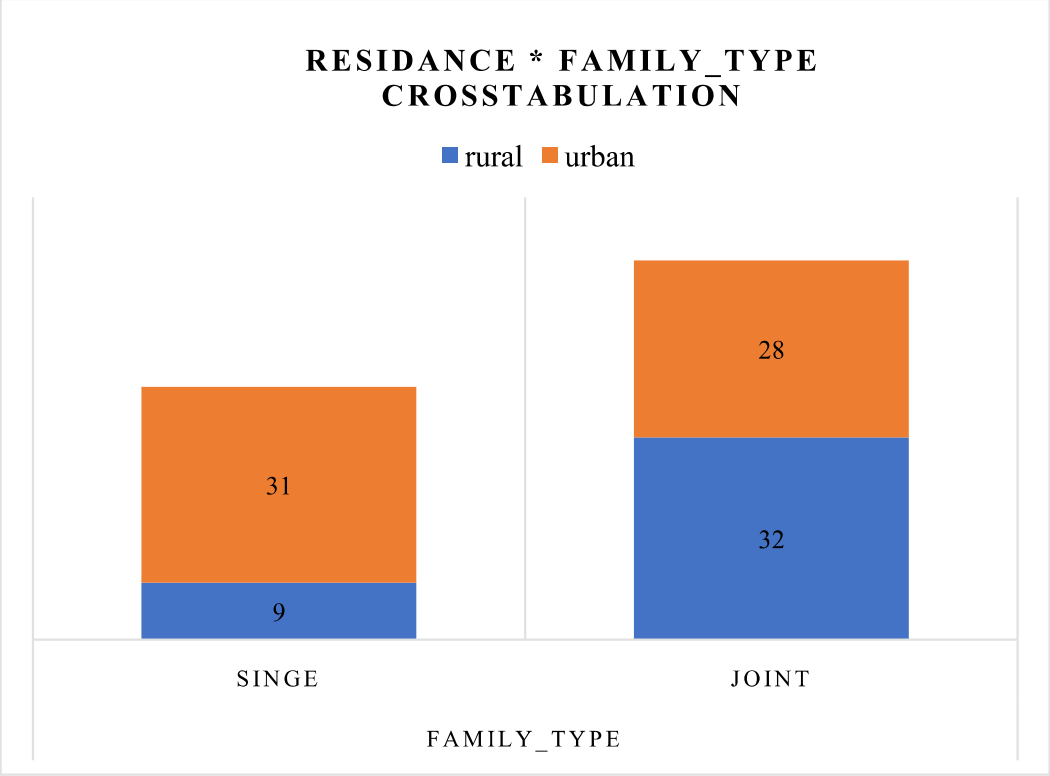

Fig. 2. Frequency distribution of residence and family type correlation

Distribution of face cover when cough or sneeze

Table 1: Frequency distribution of face covers when cough or sneeze

Sr. No.	Face cover	Frequency
1	Yes	37
2	No	63
3	Total	100

Results showed that; 63% TB patients cannot cover their face with mask when they cough or sneeze; 37 % TB patients had covered their faces with mask when they cough or sneeze as shown in table 1.

Distribution of house good ventilated

Table 2: Frequency distribution of house ventilated

Sr. No.	House ventilated	Frequency
1	Yes	79
2	No	21
3	Total	100

79% TB patient's houses were good ventilated while 21% TB patient's houses or rooms were not ventilated as shown in table 2.

Distribution of utensils separated

Table 3: Frequency distribution of utensils separated

Sr. No.	Utensils separated	Frequency
1	Yes	28
2	No	72
3	Total	100

Results showed that 72% TB patient's utensils are not separated in their house; 28% TB patient's utensils are separated in their house; as shown in table 3. There was a significant association between separated utensils and family type with prevalence of tuberculosis as p-value is <0.05.

Distribution of separate room or bed

Table 4: Frequency distribution of separate room or bed

Sr. No.	Room separates	Frequency
1	Yes	30
2	No	70
3	Total	100

Results showed that 70% TB patients had not their separate room or bed in their home; 30% TB patients had their separate room or bed in their home as shown in table 4. There was a significant association between house ventilation and family size with

prevalence of tuberculosis as p-value is <0.05.

DISCUSSION

A study was conducted to find out usual practices among TB patients. The study was conducted for 3 month duration and hospital based. Data were collected through simple interviewing method. Findings of this study showed that only 22% and 18% patients practiced correct coughing and sputum disposal techniques respectively (Cheriamane et al., 2017). Similarly current study indicated that just 37% participants cover their face during sneezing as shown in table 1.

Ventilation and incidence rate of tuberculosis are directly proportional. Pardeshi et al. performed a study in three colonies (PMG colony, Lallubhai Compound and Natwar Parekh Compound). Main objective of this study was to check out association between household structure and tuberculosis. Data were collected through questionnaire from 4080 houses. Statistical analysis showed that there was a significant association between ground floor houses and increased prevalence rate of tuberculosis (Pardeshi et al., 2020). Another study indicated that simple modifications to existing hospital infrastructure considerably increased natural ventilation, and greatly reduced modelled TB transmission risk at little cost (Escombe et al., 2019). But in this study 79% TB patient's houses were good ventilated while 21% TB patient's houses or rooms were not ventilated as shown in table 2 and there was significant association between house ventilation and family size with prevalence of tuberculosis as p-value is <0.05.

The consequences of urbanization, such as increased exposure to pathogens, have long been considered detrimental to human health. A study was conducted to analyze the relationship between urbanization and disease frequency – specifically tuberculosis. There were significant differences in survival for those with and without tuberculosis-related lesions between sites, but there were no significant differences between urban and rural sites (Kelmelis et al., 2020). In this recent study Fifty-nine percent (59%) TB patients were from urban area 41% TB patients were from rural area as shown in figure 2 but no significant association was seen between them.

CONCLUSION

Increased family size, poor hygienic environment, inadequate ventilation and low socioeconomic status were the major determinants of tuberculosis. We should provide information and create awareness about tuberculosis and it's causes so that people can protect themselves from tuberculosis. Lack of personal hygiene and knowledge related to tuberculosis were observed in patients. Poor ventilation was found as consequence of disease. We must give awareness to people about tuberculosis about unhygienic practices.

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Study on the Optimized Expression of *Pseudomonas Aeruginosa* Truncated Exotoxin A (PE38KDEL) in *Escherichia Coli*

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ABSTRACT: *The present study focused on the optimized expression of Pseudomonas aeruginosa truncated Exotoxin A (PE38KDEL) gene. This protein is considered as a potent toxin and is largely being used in the construction of immunotoxins for targeted cancer therapy. Escherichia coli BL21 DE3 Codon Plus was used as the expression host and the protein was expressed under the control of T7 promoter system. The effects of inducer type (IPTG and lactose), inducer concentration (IPTG: 0.2, 0.5 and 1 mM/lactose: 2.0, 5.0, 10, 15 and 20 mM) and incubation period (2, 4, 6, 8 and 10 hrs) were evaluated for the expressed protein. The maximum protein production was observed when IPTG at a concentration of 0.5 mM was used as an inducer however, lactose also showed substantial protein expression at 20 mM concentration. The most suitable incubation period for optimum protein expression was 6 hrs post induction.*

Keywords: *immunotoxins, promoter system, induction, lactose*

INTRODUCTION

Approximately four-fifth of dwellers on earth inhabits the less developed regions and hence 56% of newly diagnosed cancer cases and 64% of cancer deaths occurs in underdeveloped nations (Jemal et al., 2010). Consequently, cancer can be considered as a serious health risk for the people residing in Asian soils (Thun et al., 2010). It has turned out to be the primary death causal factor in Asian Pacific regions (Torre et al., 2015) and blamable for 3 million new cancer manifestations and 2 million mortal losses in Asia (Hanif et al., 2009). Pakistan ranks as the seventh most densely populated nation of the world. The political volatility, inflation and other uncertainties lead to increased incidence

of different diseases including cancer. In contrast to the past 10 years when cervical cancer was the chief death cause, now the breast cancer is responsible for utmost morbidity and mortality rates in women (Jemal et al., 2011).

New drugs to fight cancer have been and are being developed, novel therapeutic strategies are required owing to the higher frequency of drug resistance and toxic side effects resulting from the known treatments. The most challenging front in treating cancer for ages is the selective destruction of cancer cells leaving the normal body cells unharmed. Immunotherapy is a relatively new approach having the potential to combat cancer with the recently developed immunotoxins (FitzGerald et al., 2004). An immunotoxin entails an antibody joined to a toxin and is intended to explicitly exterminate tumor cells. The antibody component of the immunotoxin aims for antigens exclusive of cancer cells or profusely casing cancer cell surfaces and hypothetically spare normal body cells. The natural world harbors a vast diversity of toxins, encompassing lethal substances that are natural products of living organisms (Dosio et al., 2011). On average, only minute amount of toxin is required to mutilate cells, though the precise target and the toxic dosage may differ broadly. One of the novel therapeutic strategies being considered in hunt for promising cancer treatment relies on use of microorganisms namely live bacteria or their purified products. Microorganisms exude toxins as virulence factors in the course of pathogenic invasion or synthesize them as the products of secondary metabolism, infecting surrounding environments. Though normally unsafe and sporadically poisonous, various toxins can be exploited for therapeutic applications by modifications such as changing the method of delivery, altering the dose, reducing or synergizing particles (for instance from a heterogeneous mixture like venom). Their deadliness is heightened by the fact that these molecules hardly have any inhibitors. Diphtheria toxin (DT) secreted by *Corynebacterium diphtheriae*, *Pseudomonas aeruginosa* exotoxin A (PE), and one of the virulence factors secreted by *Vibrio cholerae*, cholix toxin are prominent examples of such bacterial toxins and share many similar structural details. Changes in molecular structure may alter the hazardous properties of a toxin converting it into a beneficial therapeutic agent (Antignani and FitzGerald, 2013). A similar strategy has been applied in case of exotoxin type A (ETA) protein of bacterium *Pseudomonas aeruginosa. Pseudomonas* exotoxin a (PE) is amongst the most potent proteins secreted by its source bacteria (gram negative aerobic bacillus) which is an opportunistic human pathogen. The remedial value of this bacterial proteins has been established by their widespread used for the construction of recombinant immunotoxins (RITs) (Pastan et al., 2006, Pastan et al., 2007, Sarnovsky et al., 2010). The aim of this study was to

express the truncated and modified toxin protein of PE38KDEL that could be used for more potent immunotoxins.

MATERIALS AND METHODS

The previously designed recombinant plasmid PE38KDEL/pET22b was used in this study. The PE38KDEL gene was designed using overlap extension PCR technique and had already been cloned into the expression vector pET22b and the positive clones were confirmed using restriction digestion and sequence analysis. The plasmid was then transformed into *E. coli* BL21 DE3 Codon Plus using calcium chloride mediated transformation method.

Expression of recombinant protein PE38KDEL

Firstly, one or two of colonies of the *E. coli* BL21 DE3 Codon Plus with positive clones and empty expression vector were grown in 10 ml LB cultures containing the appropriate antibiotics at 37ºC overnight. Next morning, 200 µl of these cultures served as inoculum for another 10 ml cultures which were allowed to grow at 37ºC at 150-170 rpm until cells reached mid log phase (OD_{600} 0.6-0.7). At this point an aliquot from the culture with positive clones was stored on ice as a non-induced control. The remainder of the cultures were induced with 0.5 mM concentration of Isopropyl β-D-1-thiogalactopyranoside (IPTG). The incubation was continued at 37ºC with aeration for 6 hrs. At various time points during the incubation period (e.g., 2, 4 and 6 hrs and overnight), 1 ml of each culture was transferred to a microfuge tube. The cells in the tubes were harvested by centrifugation at maximum speed for 1 minute at room temperature. The supernatant was discarded and the pellet was resuspended in 100 µl of 50 mM Tris-Cl buffer. After the addition of 1X Sodium Dodecyl Sulphate (SDS) gel loading buffer to the resuspended pellet, the samples were heated to 100ºC for 3 mins. The tubes were again centrifuged at maximum speed in a microfuge and the cell lysate was stored on ice until ready to load on a gel.

Optimization of the induction of target protein expression

The expression was optimized through small scale testing by analyzing the effects of various growth parameters such as; type of inducer (IPTG or Lactose), concentration of inducer and induction time. In these experiments, 10 ml of LB medium in 100 ml flasks were inoculated with freshly transformed *E. coli* DE3 colonies and the culture was placed in shaking incubator at 37ºC and 100 rpm. As the OD reached mid logarithmic phase, induction with various concentrations of IPTG (0.2 mM, 0.5mM and 1.0 mM) or lactose (2.0 mM, 5.0 mM, 10 .0 mM, 15.0 mM and 20.0 mM) for different time periods (0-10 hrs). While only one variable varied at a time with the others kept constant, 1 ml post induction samples were collected at different time intervals and bacterial growth was estimated using OD_{600}. Based on the

optical density, equivalent number of cells were pelleted, washed with 50 mM Tris-Cl buffered at pH 8.0, mixed with 1X reducing sample buffer, lysed at 100ºC for 3 mins and then finally run on 12% Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE).

The comparative distribution of expressed recombinant protein in soluble and insoluble cell fractions was also examined. The cells resuspended in 50 mM Tris-Cl were disrupted by sonication for one minute in ice box with 1-min intermittent cooling interval at 50 amplitudes (BANDELIN SONOPLUS HD2070) until the solution became translucent. Disrupted Cells were pelleted at 4ºC by centrifugation at 12000 rpm and supernatant was collected in a fresh tube. Both the supernatant representing the soluble fraction and the pellet were mixed separately with 1X SDS-PAGE Gel loading buffer, boiled for 3 mins at 100ºC and loaded onto 12% SDS-PAGE. Uninduced and induced control cell cultures were run in parallel for relative examination.

Analysis of expressed protein PE38KDEL on SDS-PAGE

The prepared samples were subjected to 12 % sodium dodecyl sulphate polyacrylamide gel electrophoresis to check the relative expression of the target protein. SDS-PAGE was carried out according to the method of Laemmli (1970). A protein gel casting system Bio-Rad was employed for the gel preparation using 8 x 10 cm glass plates. (Glass plates and combs were washed with 70% ethanol and deionized water and dried completely). The gel casting assembly was set up by placing the glass plates (separated by spacers) together and holding them with the aid of clamps. Once the gel casting system is established 15% resolving gel was formulated by mixing distilled water, 30 % acrylamide solution, 1.5 M Tris-Cl pH 8.8, 10 % SDS and 10 % ammonium per sulphate (APS) as mentioned in appendix. Tetramethylethylenediamine (TEMED) was added right before pouring the gel to initiate polymerization. The solution was swirled gently but thoroughly and appropriate amount of resolving gel was pippeted into the gap between the glass plates (leaving 1 cm vacant space at the top). To make the top surface of the resolving gel horizontal, water was filled into the gap until an overflow. Following polymerization (after 10-15 mins), 5 % stacking gel mixture was made and poured on the top of resolving gel (water discarded from above after it was gelated). Well forming comb was inserted carefully between the plates without trapping air under the teeth. The assembly was left undisturbed to polymerize at room temperature.

Once the polymerization was ensured, the comb was removed cautiously and the cassette containing the polymerized gel was placed into electrophoresis apparatus with upper and bottom reservoirs then filled with 1X Tris-Glycine buffer (wells washing was

performed in the same buffer). The prepared samples were warmed to the room temperature and 5 to 20 µl were loaded into the wells using fine tips. The gel was run at 60V for 60-80 min until the bromophenol dye reached the end of the stacking gel and then at 120V till the dye reached foot line of the glass plate.

After electrophoresis, the gel was taken out of the glass plates and immersed in Coomassie brilliant blue staining solution and slowly shaken on horizontal rotator for about 20-30 min. The gel was then transferred to the destaining solution and put on the same shaker for 2 to 3 hours until clear bands with almost no blue background were visible.

RESULTS

Protein Production

The recombinant production of foreign proteins in prokaryotic systems can be challenging and solubilization of desired protein may require a lot of time and effort. However, there is no uncertainty about the fact that production of recombinant proteins in microbial systems has transformed biochemistry. Primarily expression strains viz. *E. coli* BL21 Codon Plus (DE3) was the organism of choice for the production of recombinant protein (Fig. 1).

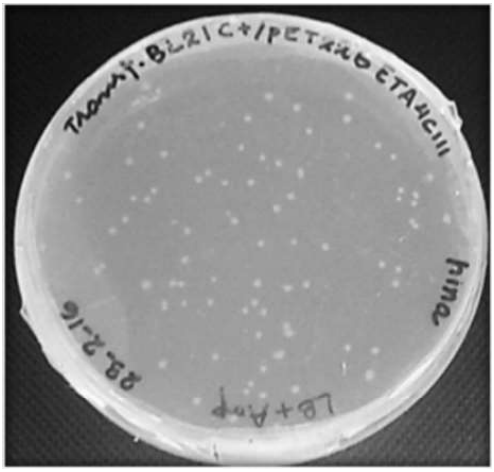

Fig. 1. *Escherichia coli* BL21 DE3 CodonPlus transformants cells on LB agar supplemented with ampicillin: White colonies observed are recombinant cells with plasmid vector PE38KDEL/pET

Expression of PE38KDEL protein in *E. coli*

E. coli BL21 Codon Plus (DE3) were transformed with PE38KDEL/pET, purified from *E. coli* DH5α and level of expression was investigated. Once the culture was grown to 0.6 OD, it was induced with IPTG at a concentration of 0.5mM and further allowed to grow for 6 hours post induction at 37 ºC. All the prepared control and induced cultures

were analyzed on 12% SDS-PAGE (Fig. 2).

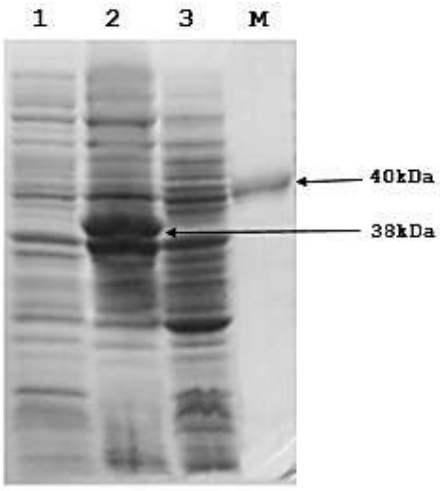

Fig. 2. 12% SDS-PAGE gel showing expression in *E. coli* BL21 DE3 (C+) harboring PE38KDEL/pET. Lane 1: Uninduced pET22b, Lane 2: PE38KDEL/pET induced (total cell lysate), Lane 3: empty pET22b (+) induced, Lane M: Protein Marker ovalbumin (40kDa)

Figure displays a notable difference in the profile of protein between the lane of bacteria with pET-22b (+) and the one with induced culture of bacteria containing pET22b/PE38KDEL. The presence of the expressed protein with the electrophoretic mobility of 38 kDa in Lane 4 confirmed that the recombinant protein was successfully produced.

Optimization of recombinant PE38KDEL/pET expression

The expression of PE38KDEL/pET was optimized using different concentrations of IPTG and lactose, post induction time and effect of host strain on expression.

Effect of IPTG concentrations on efficiency of induction

The concentrations of IPTG used to induce the lac repressor-regulated promoters can intensely effect protein expression. Keeping in view, IPTG at varying concentrations of 0.2 mM, 0.5 mM and 1 mM were tested from the suggested concentration range (0.01 mM-5.0 mM) to empirically optimize this parameter. A band of 38 kDa obtained on SDS-PAGE gel after Coomassie Blue staining R-250 in case of induced cultures confirmed the recombinant protein production. Analyzing the SDS-PAGE gel (Fig. 3), PE38KDEL expression was clearly visible at all the different concentrations of IPTG used after 6 hrs of post induction culture incubation.