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Molecular approaches and Diagnostic Strategies for Isolation and Identification of Legionella Pneumophila Serogroups 1 and 2-15 and associated Bacteria Isolated from Cancer Patients and Hospital Environment

Sanaa Saeed Atia[1], Yasin Y. Y. AL-Luaibi[2], Asaad M. R. Al-Taee[3]

[1] *College of Science, University of Basrah/ Iraq.*
[2] *College of Science, University of Basrah/ Iraq.*
Email: yasin.yousif@uobasrah.edu.iq
[3] *Marine Science Center, University of Basrah/Iraq.*

***Correspondence Author:** Yasin Y. Y. AL-Luaibi (yasin.yousif@uobasrah.edu.iq)

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Abstract

Legionella pneumophila commonly leads to respiratory infections that get acquired in the community. The pathogenesis of legionnaire's disease involves invasion of alveolar macrophages and cell mediated immunity is the primary means of immune control. Legionella pneumophila serotype1 is the most commonly reported cause of human Legionella infections. Sensitivity of urinary antigen testing is lower in immunocompromised patients because of higher likelihood of infections caused non-L. pneumophila species.

A total of 360 samples (Clinical 271 and 89 environmental samples) were collected from the Oncology Center at Al-Sadr hospital in Basrah city southern of Iraq, during January- March, 2020. (The clinical specimens included blood, urine and sputum, were taken from patients attending and /or admitting to the center. Meanwhile, the environmental samples were collected from air conditioners, hospital toilets and water). According to the morphpological features of L. pneumophila on Buffered charcoal yeast extract (BCYE) agar, and using 16S rDNA gene sequence revealed that forty seven species have been obtained in addition to95 legionella spp out of 47 species, 36 species belong to Bacillus cereus and B. anthracis, one genus of Alcaligenes aquatilis, Enterococcus sp., Klebsiella oxytoca, Escherichia sp. and Serratia sp.,in addition to4 genus of Pseudomonas sp. and 2 genus of Staphylococcus haemolyticus. All these isolates were isolated from samples of blood, urine, sputum, water and air.

The idenfied Legionella spp. were more characterized using six specific primer. Using JFP and JRP primers, When the second specific primer L5SL9, L5SR93 were applied, the result showed that all isolates were positive. However, applying the third specific primer dnaJW19F, dnaJW19R for differentiate between all L. pneumophila serogroups and the strains that belong to the various species of Legionella.It should be mentioned that the Legionella bacteria physiological tests were carried out in a separate study simultaneously with this one, pending its publication. The following tests are among them: using biochemical tests and some physiological experiment, which include: catalase, oxidase, Dnase, gelain liquefaction, hippurate hydrolysis, urease, biofilm forming (tube and Congo red methods), tissue culture plate method, starch hydrolysis, citrate utilization, himmagglutination activity, protease production, effect of pH, temperature and salinity and lecithinase and lipase production accomplished by serotyping the serogroup of Legionella pneumophila was performed using Hi Legionella Latex Test Kit, the identified legionella spp. subjected to nine types of antibiotics to determine the susceptibility which included; azithromycin 15 µg, cefotaxime 30µg, ciprofloxacin 5µg, doxycycline 30 µg, erythromycin 15 µg,

levofloxacin 5µg, ofloxacin 10 µg, norfloxacin 10 µg and Rifampicin 5 µg. antibiotics were subjected to detect minimum inhibitory concentrations using MIC strips which including azithromycin 0.016-256 mcgmL-1, cefotaxime 0.002-32 mcgmL-1, ciprofloxacin 0.016-256 mcgmL-1 and levofloxacin 0.002-32 mcgmL-1.

Keywords

Legionella Spp., Cancer Patients, Legionella Serogroups, New Strains.

Cancer is a disease in which a group of abnormal cells repeatedly divide uncontrollably while disobeying the normal rules of cell division (Bekele, 2022). Cancer is a widespread health issue brought on by poor lifestyles and genetics. Pathogenic infections are now more frequently recognized as initiating or promoting human cancer. The majority of the bacteria, viruses, fungi, and parasites linked to human tumors can also cause the development of cancer. The host cell activity is further altered by the microorganism's gene products or protein release, leading to aberrant cell formation and proliferation. Several bacteria have been identified as specific biological agents that cause cancer because of their features that cause cancer (Rahman *et al.*, 2022). Infection problems remain the main causes of morbidity and mortality in cancer patients, despite improvements in long term survival brought about by advancements in treatment and supportive care for cancer patients. (Li *et al.*, 2021; Babady, 2021). In the current study Due to its widespread respiratory diseases and their overlapping symptoms, there is a need to deeper understanding the study of this bacterium, which is rarely discussed locally, particularly given that it causes serious respiratory diseases, particularly those that coincide with cancerous diseases, and that patients suffer from continuous and various treatments in the absence of high immunity. The aim of the current study can help achieve by, isolation and identification of *Legionella* bacteria and associated bacteria from cancer patients and hospital environments , the use of diagnostic methods represented by using of molecular genetics applications approved for diagnosis, and investigating the appropriate antibiotics is especially important since the limited vision diagnosis leads to prescribing a treatment that does not affect the pathogen and even leads to the accompanying side effects. The presence of bacteria in cancer patients is attributed to the noticeable decrease in the level of immunity among patients, the use of contaminated tools represented by contaminated catheters, urine bags, door handles, bathroom faucets and water tanks, as well as the most important role due to the irregular use of medicines, which in turn creates new generations of bacteria resistance, Legionnaires' disease (LD) was Infections in humans arise exclusively by the inhalation of aerosols that contain *Legionella*, which can occur in air conditioning systems, cooling towers, spas, fountains, ice machines, plant sprayers, dental appliances, and showerheads(Borges *et al.*,2016). The current study aimed to focus in general on the emergence of *Legionella* in particular in cancer patients which is attributed to the low level of immunity in cancer patients, as well as to a pattern of hidden growth of this type of bacteria inside the cells, which is difficult to treat in addition to isolate other associated bacteria with *Legionella* spp.

Materials And Methods

Clinical specimens and Environmental specimens:

A total of 360 samples were collected from the Oncology Center at Al-Sadr hospital in Basrah city. Clinical samples were included 271, in addition to 89 environmental samples, during January- March, 2020. The clinical specimens included blood (23), urine (19)and sputum (39), were taken from patients attending and /or admitting to the center whom suffering from different types of cancer such as breast, lung, osteosarcoma, prostate, ovary, pancreatic, stomach, colorectal, liver, bladder, uterus, and kidney. Meanwhile, the environmental samples were collected from air conditioners(9), (24) airsamples, hospital toilets(23) and water(33) .

Culture:

Three different types of culture medium, including Nutrient Agar, Blood Agar, and Buffered charcoal yeast extract (BCYE) Agar, were used to cultivate each sample. The growth of colonies was noticed during the incubation for 24-48 h. at 37 °C. These colonies were first identified microscopically, which led us to isolate them on a plate. One hundred isolates that are most likely Legionella were more characterized from 360 samples. The suspected Legionella were culture on buffered charcoal yeast extract agar plates supplemented with L-cysteine and ferric chloride, whereas Bacteria from other genera were incubated on nutrient agar at 37 C for1–3 d depending on the species.

It is worth noting that many physiological tests were conducted, such as catalase and oxidase tests, DNase test, numerous physiological, biochemical and immunological tests were prepared (data under publishing), in addition to Antibiotic susceptibility Also, a number of physiological experiments were conducted. However,after that the molecular approach was applied to distinguish between Legionella pneumophilaand other Legionella spp. in addition to further of different bacterial species rather than Legionella.

DNA extraction:

Overnight cultures were grew, one bacterial colony incubated in 5 mL brain heart infusion broth, then transferred to 1.5 mL micro eppendorf tubes and washed three times using normal saline with centrifuging for 5 min at 13000 rpm. The DNA was extracted from bacteria obtained from clinical and environmental sources according to Presto™ Mini gDNA Bacteria Kit ,(Geneaid –Tiawan). The extracted DNA was either amplified immediately or stored at −20°C for up to 6 months until analysis.

Molecular characterization of bacterial species isolated from cancer patients and the hospital environment associated with *Legionella*

16S rDNA Gene Amplification

A molecular identification of the bacteria was performed by amplifying the *16S rDNA* gene using the universal bacterial oligo-nucleotide primers 27 Forward: 5`-AGAGTTTGATCCTGGCTCAG-3`and 1492R reverse: 5`- GGTTACCTTGTTACGACTT-3` .This universal 16s rDNAand other specific primers used in the current study were provided from macrogen company /Koria.The PCR conditions were:Five microliters of extracted template DNA was used in a 50- µl reaction mixture that included 2 µl primer 27F, 2 µl primer 1492R, 25 µl of Mastremix (Promega company/ USA) ,16 µl of nuclease free water.Thermal cycling was performed with a *AllInOneCycler*™ (KR10-1343891) Bioneer /Korea . An Initial denaturation 94°C for 5 min, 35 cycles consisting of denaturation 94°C for 30 sec,annealing temperature at 55.5°C for 30 sec,extention 72°C for 30sec, were followed by a final extension at 72°C for 5 min . Gene amplified were detected using 2% gel electrophoresis and visualized in transilluminator.The amplified gene sent for sequencing in Bioneer company/ Korea. **The PCR product detection for this gene** was: Six microliters of PCR product were loaded in the well. and the mixture was electrophoresedon 2% agarose gels in TBE buffer (5 mM Tris, 5 mM boric acid, 0.1 mMEDTA) with ethidium bromide (0.5 mg/dl) at 70V for 45 min. The gels were viewed under UV light. Four microliters of 100 bp DNA ladder were loaded in the first well of the gel. The reaction was run for 1 h. The band of amplification was with size 386 bp then it was photographed under UV light, after that , PCR products were stored at -20 ° Cfor further investigations.

Identification of Bacterial Species :

The upcoming sequences of 16S rDNA gene sequence were analyzed in the Basic Local Alignment Search Tool (BLAST) to look for homologous sequences in the National Center for Biotechnology Information database (NCBI) http://www.blast.ncbi.nlm.nih.gov. The bacterial sequence was identified by matching it with a sequence with the highest identity score from the GenBank database (Jenkins *et al.*, 2012).

Amplificationof part of 16S rRNA using Specific Primers for *Legionella* Spp.

1. JFP gene primers:

The 16S rDNA gene of suspected *L. pneumophila* was mainly amplified using *JFP* primers which amplify from base 451 to 837 .*JFP* (5`-AGGGTTGATAGGTTAAGAGC-3`) and *JRP* (reverse primer) (5`- CCAACAGCTAGTTGACATCG-3`) .**The PCR conditions for this gene** was: Five microliters of

extracted template DNA was used in a 50-ml reaction mixture that included 2 µl primer *JFP*, 2 µl primer *JRP*, 25 µl of AccuPower® PCR PreMix completed by 16 µl of nuclease free water. Thermal cycling was performed with a *AllInOneCycler*™ uses the patented (KR10-1343891) Bioneer / Korea). an Initial denaturation 94°C for 5 min, and followed by 35 cycles consisting of denaturation 94°C for 1min, annealing temprrature at 57.3°C for 1:30 min,extention 72°C for 1:30min, and then followed by a final extension at 72°C for 5 min .

2. Detection of L5SL9 and L5SR93 genes:

The primers *L5SL9* (5'-ACTATAGCGATTTGGAACCA-3') and *L5SR93* (S'-GCGATGACCTACTTTCGCAT-3'), which have been designed to generate a 104-bp fragment from the 5s RNA gene of the genus *Legionella*(Mahbubani *et al.*,1990). Genes were amplified by PCR for bacterial samples according to Alexiou-Daniel *et al.* (1998). It should also be noted that the primer was applied to 95 isolates.**ThePCR conditions were:**

Five microliters of extracted template DNA were used in a 50-ml reaction mixture that included 2 µl primer *L5SL9*, 2 µl primer *L5SR93*, 25 µl of AccuPower® PCR PreMix ,16 µl of nuclease free water.Thermal cycling was performed with a *AllInOneCycler*™ (Bioneer). The programe was included an Initial denaturation 94°C for 5 min, followed by 30 cycles consisting of denaturation at 94°C for 30 sec, annealing at 58.6°C for 30 sec, extention 72°C for 30min, were followed by a final extension at 72°C for 5 min .The PCR products were electrophoresed on a 2% agarose gel as described above .The size of the amplification band for positives was 104 bp. The PCR results were then kept at -20 °C for further analysis.

3. Detection of W 19F(dnaJ) and W 19R (dnaJ) genes :

For more identification for L. pneumophila serogroups and members of the Legionella genus. dnaJ gene were applied which is more variable than the 16S rDNA sequences. The W 19F(dnaJ) is an amplicon of 285 bp. Thus, W 19F(dnaJ) 5'-AGGTGGTTTTGGCGGATTTGG-3' and W 19R (dnaJ) genes 5'-TGAATTCTGACTTGCCCCATG-3' were amplify this gene by PCR, according to Liu et al. (2003) .The sequences of primers as above . With the exception of the annealing temperature, which was 55.5 for 30 seconds, the PCR conditions are the same as those described for the previous primers. The PCR products for W 19F(dnaJ) and W 19R (dnaJ) was visualized as described previously using 2% agarose gel.

Statistic Alanalysis

The statistical analysis was performed using Chi-squared test in SPSS program (Version 26), a P value $\leqslant 0.05$ was considered as statistically significant and $P \leqslant 0.01$ was considered as highly significant.

Results

Out of 360 samples were collected from the Oncology Center at Al-Sadr hospital in Basrah city. And after doing biochemical ,physiologicaland immunological test(data under publishing) to identify *Legionella* spp. and associated bacteria from cancer patients and the hospital environment, 150 isolates were subjected for DNA extractionand molecular identification using 16S rDNA, and other Three specific genes. Out of 150 isolates, 95 were identified as *Legionella* spp. and 47 were associated species.

16S rDNA Gene Amplification

Of the150 isolate forty seven isolates were applied for 16srDNA amplification.The results of amplification of *16S rDNA* gene from bacterial isolates are shown in Figure (1).

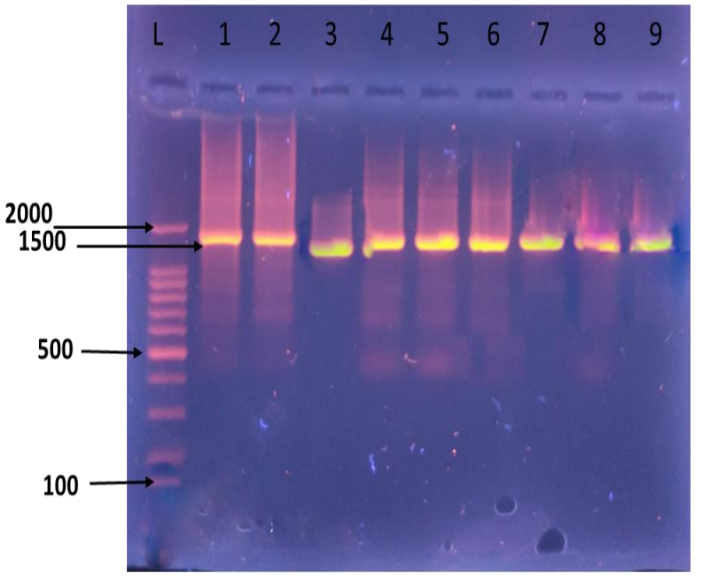

Figure 1: Amplification of *16S rDNA* gene bacterial isolates as showed using a 2 % agarose gel containing ethidium bromide: Lane L: 1kb molecular weight DNA ladder (promega/USA) , Lane 1-9: *16S rDNA* gene bands of bacterial isolates

Sequencing of 16S rDNA gene

The results of *16S rDNA* gene sequences after data manipulation for diagnostic purposes were showed that the total number of samples were 47, of which it was recorded; One sample for each, *Alcaligenes*, *Enterococcus, Escherichia, Klebsiella,* with *Serratia*. The percentage of each separately was 2%. As for *Bacillus,* the results showed its presence in 36 samples (75%), *Pseudomonas* was present in 4 samples (8%), and *Staphylococcus* two samples (4.2%). The sources of isolation of these bacteria mentioned above, (was also obtained one sample (2%) from the air-conditioned hospital environment,15 samples (31.3%) from the hospital environment / bathrooms, air, the number of sputum samples was 11 samples (23%) and finally the number of urine samples are 2 (4.2%),and 18 samples from blood (37%) .

Identification of new local bacterial isolates

Sixteen new local bacterial isolates were identified by comparing nucleotides sequence with their type strains. The new strains databases were recorded in DNA Data Bank of Japan (DDBJ) and published in The National Center for Biotechnology Information (NCBI) and the Gene bank.The Frequency of the New local isolates in Clinical and hospital environment isolates that were registered as new strains, their total number was 16, of which as it was obtained 7 samples(6%) from the hospital environment, and 9 samples (4.3%) from clinical samples, with a significant differences between total isolates and a new strains .

PCR amplification using Specific primer :JFP and JRP for Legionella detection :

The Specific primer *JFP* and *JRP* were used in the current study to amplify a specific part from *16r DNA* in *Legionella* spp. A 386 bp portion of the whole genome is amplified by this primer As showen in Figure(2). The results of the using this primer, which was applied on 95 isolates, showed that only 5 isolates were negative, with a rate of 5.3%, whereas 90 isolates were positive, (94.7%), with asignificant differences $P \leqslant 0.01$, as demonstrated in Figure (2).

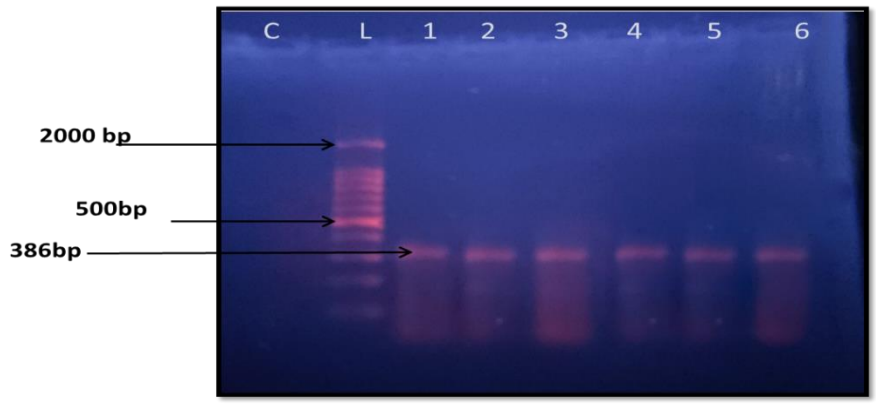

Figure 2: The gel detection of PCRamplification using *JFP* specific primer with product size 386 bp as showen ,c:control *E.coli*,L:Lader ,1,2,3,,5 and 6 *Legionella pneumophila* 2-15 , *Legionella pneumophila* 2-15, *Legionella pneumophila* 2-15, *Legionella pneumophila* 1, *Legionella pneumophila* 1 and *Legionella pneumophila* 1 respectively .

PCR amplification using Specific primer : L5SL9 and L5SR93

The Specific primer *L5SL9* and *L5SR93* were used in the current study to amplify a specific part from 16srDNA in *Legionella* spp. A 104 bp portion of the whole genome is amplified by this primer,the results of the statistical analysis of the trial of using the aforementioned primer, which was conducted on 95 isolates have showed that all isolates bands at size 104 bp as demonstrated in Figure (3).

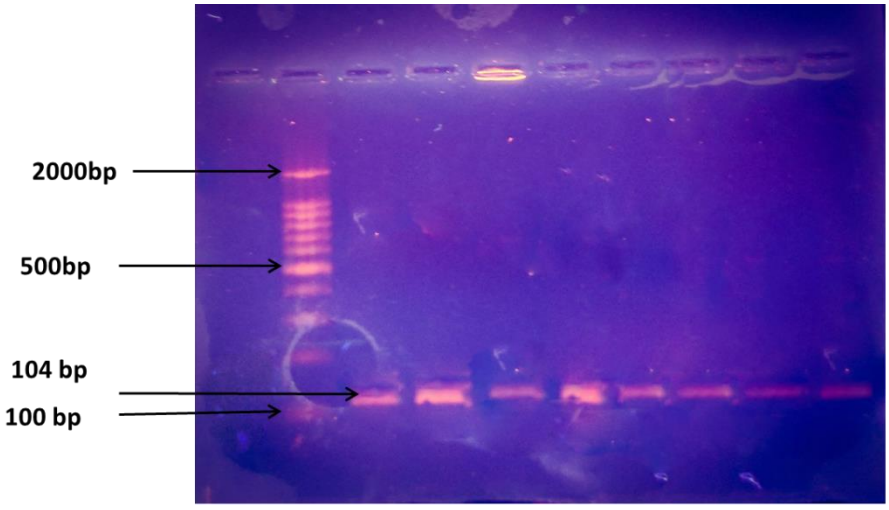

Figure 3: The gel detection of PCRamplification using *L5SL9* and *L5SR93* specific primer with product size 386 bp as showen in c:control *E.coli* , L:Lader ,1,2,3,4,5 , 6,7 and 8 *Legionella pneumophila* respectively .

PCR amplification using Specific primer : dnaJ W19F and dnaJ W19R

The Specific primer *dnaJ* W19F and *dnaJ* W19R were used in the current study to amplify a specific part from 16s ribosomal DNA in *Legionella* spp. A 285 bp portion of the whole genome is amplified by this

primer. At this amplification which was conducted on 95 isolates. The results showed that it has been obtained 16 isolates were negative to this amplification with a percentage of 16.6% represented by 4 isolates from blood samples, one isolate from sputum samples, 4 isolates from air samples, and finally 7 isolates from tap water samples. Whereas 80 isolates were positive with band size 285 bp as demonstrated in Figure (3), with a percentage of 83.3%, and significant differences appeared, as demonstrated in Figure (4).

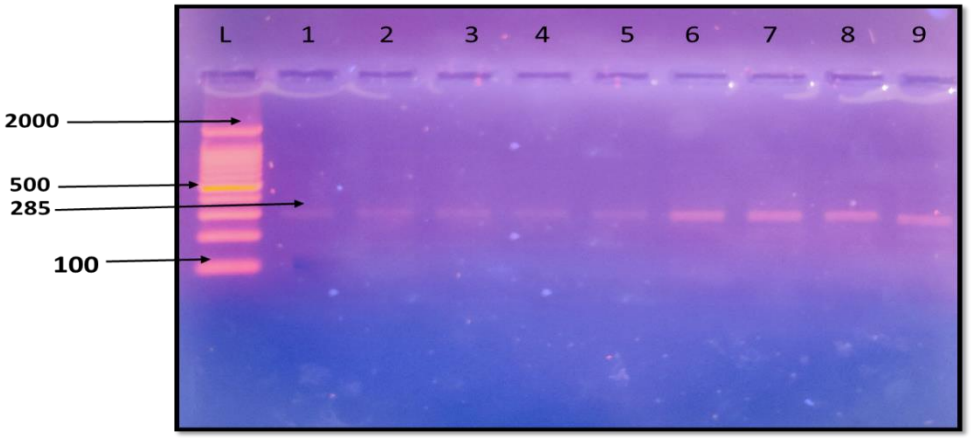

Figure 4 : The gel detection of PCRamplification using *JdnaJ* W19F and *dnaJ* W19R specific primer with product size 285 bp as showen,L:Lader ,1,2,3, *Legionella pneumophila* 2-15 and 4,5,6,7,8 and 9 *Legionella pneumophila* 1 , respectively .

Discussion

Identification of bacteria using the 16S rRNA gene:

By employing the F27 and R1492 primers in a PCR experiment, the bacterial 16S rDNA gene was identified. Since this marker is applicable to all known types of bacteria, it can be utilized as a trustworthy tool for the identification of bacteria. By comparing the sequence of the type strain from the gene bank, 47 of the bacteria were successfully identified to species level. According to the sequence of the current study, *Bacillus* was the most prevalent genus, with 36 isolates (75%). Of these, *Bacillus cereus* represented 24 isolates, with 11 of them isolated from blood samples, 11 of them from sputum sources, and 5 of them from hospital environment. There are 10 isolates of *Bacillus anthracis*, 5 isolates recovered from each of *Bacillus licheniformis* and *Bacillus subtilis* from blood sources, three isolates from sputum, and one from a hospital environment. This finding is in agreement with numerous research studies Al-Habibi *et al.*, (2022)and Mbhele *et al.*, (2021),where the *Bacillus* species is the most common bacterial agent in charge of food borne outbreaks and a well-documented pathogen that causes nosocomial bloodstream infections. In order to point the climatological factors connected to *Bacillus* positive blood cultures, a detailed analysis of *B. cereus* strains from nine hospitals, isolated from patients and healthcare settings was carried out over the course of a five year study, as noted by Glasset *et al.* (2018). The diverse pathogen *Pseudomonas aeruginosa* is linked to a variety of human illnesses. The bacteria is a significant source of infection in healthcare settings, especially in those at risk, such as those with burns, neutropenia, or undergoing critical care. These populations may have excessive fatality and morbidity rates linked to infections with *P. aeruginosa*. Managing infections can be difficult since *P. aeruginosa* is intrinsically resistant to several medications. (Kerr and Snelling, 2009). Four isolates of the genus *Pseudomonas* were also acquired for the current study, which likewise yielded findings. Four isolates, or 8.3%, with two of the four being ascribed to *Pseudomonas aeruginosa* in Blood is their source, and the isolate that follows them is *Pseudomonas gessardii*, which originates from the same place. This finding is consistent with those of numerous researchers who have stated that *P. aeruginosa* is a pathogen with a wide range of abilities (Paprocka *et al.*,

2022; van der Zwet *et al.*, 2022; Bhat *et al.*, 2021; Kerr and Snelling, 2009). While *Pseudomonas fluorescens* was isolated from clinical sources. The number of infections linked to this bacterium is extensive and keeps expanding. Some illnesses are more likely to occur in communal settings and have an inherent resistance to numerous types of antibiotics, which restricts the range of available treatments.It is not remarkable that attempts are made to detect *P. aeruginosa* in the hospital environment, particularly in water sources, sinks, taps, and showerheads, given the bacterium's broad dispersion..

In the current study, two isolates belonging to *Staphylococcus haemolyticus* were also obtained, which account for 4.2%; one of them was isolated from a blood sample, and the other was isolated from a sputum sample. The result is consistent with Talagtag *et al.*, (2021). It is a typical human pathogen that can cause a variety of illnesses in both the community and hospital settings, including skin and soft tissue infections, abscesses, pneumonia, osteomyelitis, endocarditis, arthritis, and sepsis.

The *Alcaligenes* genus is a member of the *Burkholderiales* order's Alcaligenaceae family (Garrity *et al.*, 2005). *Alcaligenes aquatilis* one isolatewas obtained in the current study which accounts for 2.1 %. which was isolated from the hospital environment. This result is in agreement with(Usman *et al.*, 2021). *A. faecalis* being the main isolate found in hospital waste water samples analyzed, *S. saprophyticus* was next, with levels beyond those permitted by the WHO, HPA, EPA, and FAO. The analysis of hospital wastewater samples revealed a significant incidence of drug-resistant isolates, indicating their tenacity in the hospital setting and capacity to spread antibiotic resistance (Usman *et al.*, 2021). In the present investigation, only one isolate of *Enterococcus faecalis* was identified. As a cause of oxidative stress. An imbalance between the body's antioxidant system and the generation of active oxygen radicals leads to oxidative stress. Cancer formation is greatly influenced by oxidative stress. The colon epithelial DNA is damaged by reactive oxygen radicals produced by *Enterococcus faecalis.* Adenomatous polyps and colorectal cancer are caused by reactive oxygen radicals (Aribisala and Sabiu, 2022). Cancer patients are vulnerable to Blood stream infections (BSIs) for a number of reasons. The bloodstream can be more easily accessed by bacteria and fungus due to changes in anatomical barriers. In addition, different diseases and tumor types have distinct effects on the spread of microorganisms and the risk factors for BSI. (Bai *et al.*, 2022). *Klebsiella oxytoca* and *Escherichia coli* were also among the isolates identified in the current study, both of which were isolated from urine, accounting for 2.1% of each. This result is consistent with what was stated by Shrestha *et al.*, (2021); Bhat *et al.*, (2021)and Strakova *et al.*, (2021) that bacterial urinary tract infections that are resistant to antibiotics are more frequent in cancer patients (UTIs).

The findings of the present study supported previous reports (Nguyen *et al.*, 2022; Pérez-Viso *et al.*, 2021) thatstated a just one isolate of *Serratia marcescens*, accounting for 2.1%, was present. *Serratia* species are common in nature; they can be found in both water and soil. They are related to soil as well as to plants, insects, people, and other animals. *Serratia marcescens* is the species that infects people most frequently among these, even among preterm infants.

Identification of New local isolates

Sixteen local isolates of by comparing nucleotides with their type strains, several bacterial strains were distinguished.. (1% difference in 16S rDNA sequence) from the blood, urine, and sputum of cancer patients or hospital environments were reported as new local isolates in the current study , Since classification frequently relies on a range of 0.5% to 1% difference (99.5% to 99% similarity), as demonstrated by the findings of various research taken together (Abd Al-abbas, 2012b). These new local isolates may be the result of a their passage from one habitat to another caused them to mutate.

The alteration could be caused by errors in the process of DNA replication, including insertion or deletion (Aminetzach *et al.*, 2005). In the current study, 7 (6%) local isolates were obtained out of 16 new local isolates sourced from the hospital environment and 9 (4.3%) local isolates out of 16 new local isolate collected from clinical samples (sputum and urine), and it should be noted that all sources

of samples are from cancer patients or hospital residents. The new local isolates of the present study may be due to the fact that the bacterial species were exposed to different types of chemicals, like a wide range of special antibiotics for mutant samples and microbicides, which are broad spectrum chemical agents that inactivate microorganisms. They are frequently used in industrial, household, and healthcare settings where their applications include antisepsis, hard surface disinfection, and pharmaceutical product preservation. They may also be incorporated into medical device coatings, for instance in sutures, wound dressings, and urinary catheters, to inhibit bacterial adhesion and subsequent biofilm formation (Cowley *et al.*, 2015; Tag ElDein *et al.*, 2021).

In the current study the reason behind not using the bacterial 16S rRNA gene in the diagnosis of *Legionella* bacteria Rather, a specialized gene with a specific sequence was used from the total genome of bacteria, this is consistent with Cloud *et al.* (2000)due to A BLAST search of the 386-bp PCR product from the first eight samples with discrepant results showed that they contained sequences with homology to the 16S rRNA gene of*Acinetobacter* species or an unidentified *Proteobacterium* 16rRNA gene and does not give any indication that they are*Legionella* species. The sequence in each of the remainingsamples with discrepant results was homologous to those of *Legionella* species and had fewer base pair differences from *L. pneumophila* , The other reason lies in A PCR assay was developed in which the target for the testis the 16S rRNA gene, which exists in multiple copies pergenome, thus improving the sensitivity of detection. Severalmedically relevant *Legionella* species including *L. pneumophila*,

L. micdadei, *L. bozemanii*, *L. longbeachae*, *L. feeleii*, and*L. dumoffii* can be detected without the use of culture. Thisstudy compares PCR to culture with respiratory samples frompatients suspected of having *legionella* infection Cloud *et al.* (2000).

Specific primer :JFP and JRP:

PCR is performed with primers previously determined to amplify a 386-bp product within the 16S rRNA gene of *Legionella pneumophila.*

We can specifically detect the clinically significant Legionella species including L. pneumophila, L. micdadei,L. longbeachae, L. bozemanii, L. feeleii, and L. dumoffii Cloud *et al.* (2000).The 386-bp amplification product being detected is a successful outcome for the current legionellae PCR screening. In the current study, out of 319 samples, 100 were examined using the PCR technique to detect *Legionella.* The results obtained were 5.3% negative and 94.7% positive. This primer was applied to the samples that actually gave a positive result growth on the BCYE medium. This result is in agreement with Cloud *et al.* (2000), who showed that of the 31 samples reported as culture positive, all were also positive by PCR, although it was performed with DNA amplicons from 12 samples that were PCR positive but culture negative. while Vittal *et al.* (2021) reported that all samples that testedwere positive for .

Specific primer : L5SL9 and L5SR93 for Legionella spp. :

In the current investigation, all 95 samples were analyzed for this gene gave apositive results . As the outcome of the present research was in well beingt with Andreozzi *et al.* (2016) and Mahbubani *et al.* (1990), where the researchers showed that of *Legionella* have a 104-bp DNA sequence that codes for a component of the 5S rRNA *Legionella*, including all 15 serogroups of *L. pneumophila* that were examined.

Specific primer : dnaJ W19F and dnaJ W19R:

The *W 19* (*dnaJ*) (285 bp) was used as quick identification technique and detecting members of the *Legionella* genus and *L. pneumophila* serogroups (*dnaJ*). As a result, the conclusions of the present study results that the *dnaJ* gene is a helpful tool for identifying the species of *Legionella* and *L. pneumophila* serogroups. A negative (15.8%) and a positive (84.2%) result were achieved. This is consistent with what claimed about creating a PCR detection primer for *L. pneumophila* and other *Legionella* species (Liu *et al.*, 2003). For *Legionella pneumophila*, *w19* was used as a detective primer set which produced a 285 bp amplicon.

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