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Effect Of Different Temperature On The Pseudomonas Aeruginosa Biofilm Formation

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Abstract:
Pseudomonas aeruginosa is a gram-negative bacillus and motile in nature, it is an opportunistic bacterium and causes several types of infection especially nosocomial infection in old age and children are most commonly infection. It was observed that their growth and development mainly depend on biofilm formation which makes it more tough and more survivor in adverse condition. The three main factors which has great impact on biofilm formation are pH, Temperature and salinity. It was observed from many experiments that p. aeruginosa can adapt its adverse condition after certain period of time and can start growth on its own. In the experiment based on different temperature 4 , 25 and 45 respectively show different biofilm formation. It was observed that the biofilm formation of p. aeruginosa at 4 and 25 formed.

Keyword: *p. aeruginosa, temperature, biofilm, nosocomial, infection*

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I. Introduction

Pseudomonas aeruginosa stands as an enigmatic force in the microbial world, exhibiting remarkable adaptability and a diverse array of virulence factors enabling its survival across various environmental niches. This Gram-negative, rod-shaped bacterium is characterized by its unique traits, including its pearlescent appearance, grape-like or tortilla-like Odor, and growth versatility within a broad temperature range (25°C to 37°C), distinguishing it from other Pseudomonas species. Notably, its ability to thrive at higher temperatures (42°C) enhances its survival capability. Its presence is ubiquitous, exhibiting a proclivity for diverse environmental conditions and demonstrating pathogenicity across plants, animals, and notably, humans, especially in immunocompromised individuals. P. aeruginosa orchestrates its pathogenicity through a sophisticated suite of virulence factors and a complex regulatory network, finely tuned to ensure its survival [1, 2, 3, 4, 5].

These mechanisms encompass a repertoire of adhesins, secretion systems, and toxins, each playing a pivotal role in its interaction with host cells and establishment of infection. The bacterium utilizes various adherence factors such as type IV pili, flagella, and the core oligosaccharide of lipopolysaccharide (LPS) to adhere to host cells, facilitating colonization and nutrient acquisition. Type IV pili-mediated adhesion, in particular, constitutes a significant portion of its adhesion capability to eukaryotic cells, interacting with specific cell surface components to promote attachment. P. aeruginosa employs an array of secretion systems, including type I, type II, type III, type V, and type VI, to deliver an assortment of toxins and exoenzymes. These toxins, such as Exotoxin A, elastases, alkaline protease, phospholipase C, and rhamnolipids, exhibit diverse activities affecting host cells' physiology, cell signalling [6, 7, 8, 9, 10].

These effectors play crucial roles in manipulating host cell signaling pathways, leading to programmed cell death or apoptosis, which can be both advantageous and detrimental to the host depending on the infection context. P. aeruginosa exhibits remarkable adaptability conferred by its extensive genomic repertoire and regulatory genes, enabling it to respond to various environmental cues and intricately regulate the expression of virulence factors for optimal survival [11, 12].

II. Method And Material

Site selection for the present study

All the clinical samples were collected from urology care clinic, Bariatu, Ranchi, Jharkhand and non-clinical items were collected from drainage system of Bariatu.

Collection of Samples

All the samples were collected from the patients who were suffering from infection (urine, Pus, Blood etc.) and from the surfaces of the hospital which includes food tray and lift bottoms. Media were first autoclaved at 121 for 15 mints for 15psi to avoid any kind of contamination to the media. Once the media gets solidified the selected samples were swabbed or streaked on the MacConkey agar petri plate and incubated at 37 for 24 Hrs. after incubation the colonies were selected on the bases of color of the colony white non fermenting and pink fermenting bacteria. As the MacConkey agar is known as differential media.

Preparation of the cultural media

Isolation of Pure Culture

The selected colonies were selected from the MacConkey agar media and then transferred to the Nutrient agar media. The inoculation was done aseptically. The Nutrient agar media was first prepared according to required quantity and then it was autoclaved at 121 for 15 minutes at 15psi. Then it was poured in the petri plate and it was left for the solidification, once the solidification was done the cultures were inoculated and incubated for 24 Hrs. at 37 . Then the cultures were morphologically examined and observed.

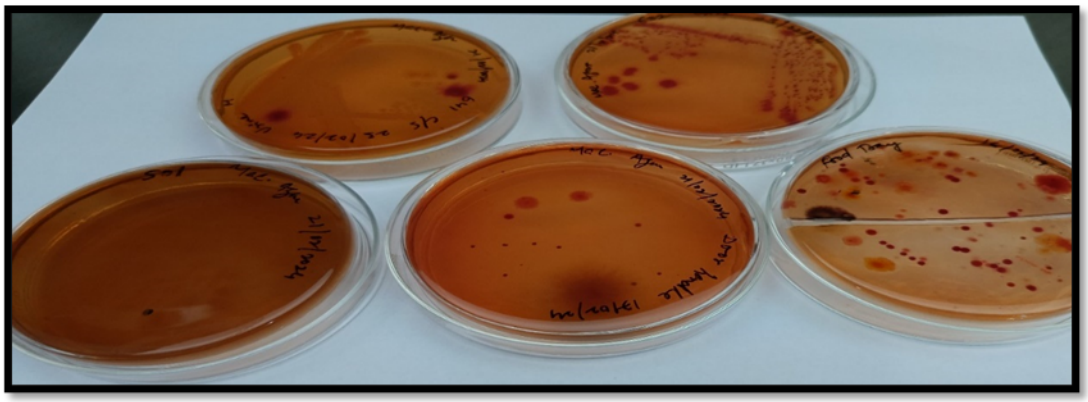


Figure 1: Samples collected from clinical and non- clinical sources on MacConkey agar

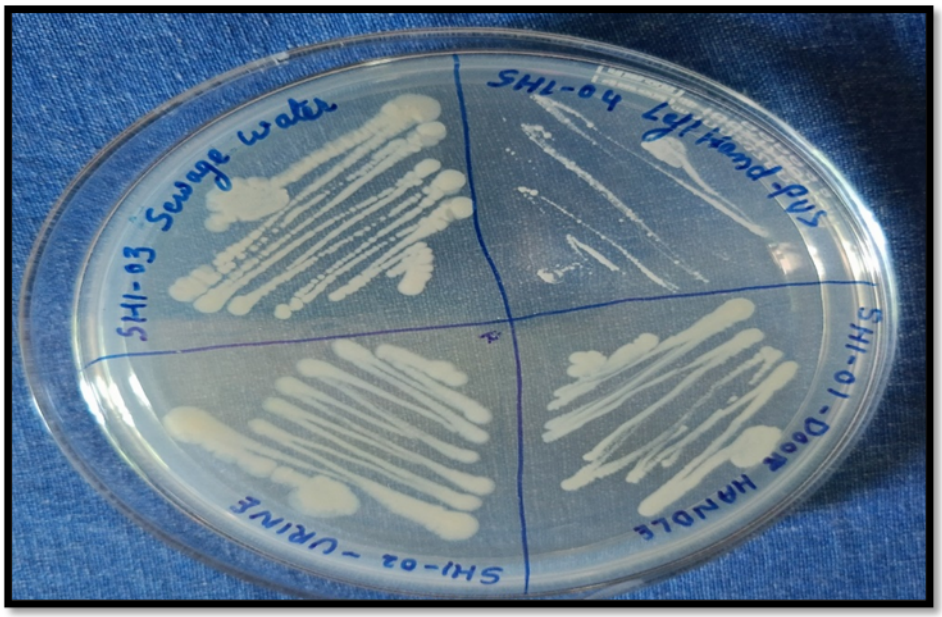


Figure 2: Growth on Nutrient agar under Optimal condition

Morphology of the isolates:

After 24 hours of incubation the isolates were observed for the selection of the colonies the isolates were smooth and rough, irregular margin, flat elevation, opaque, some colonies were white, off white and some shows pigmentation. On the MacConkey agar colonies were grow white in color as it can't ferment the lactose. Due to which MacConkey agar is also known as differential media.

Table 1: Isolates shows morphological characteristics on Nutrient agar

Isolates	Size	Pigmentation	Form	Margin	Elevation	Optical Character	Shape	Gram Stain
SHI-1	Large	Off-white	Irregular	Rough	Flat	Opaque	Short Rod	-ve
SHI-2	Large	Off-white	Irregular	Rough	Flat	Opaque	Short Rod	-ve
SHI-3	Moderate	Off-white	Irregular	Rough	Flat	Opaque	Short Rod	-ve
SHI-4	Pin Point	Off-white	Irregular	Rough	Flat	Opaque	Short Rod	-ve
SHI-5	Pin Point	Off-white	Irregular	Rough	Flat	Opaque	Short Rod	-ve

Gram Staining

The gram staining is one of the most important methods in the field of bacteriology which helps to identify the bacterial identification and observation throughout experiments. It is also the first most important test to be performed. There were three different stains were used primary stain Crystal Violet and secondary stain Iodine followed with decolorizing agent and counter stain as Saffranine. After application of the gram staining isolates shows pink color which means its loses primary stain after application of the decoloring agent and takes only counter stain i,e saffranine due to which it appears pink in color. It is because it has outer membrane which is absent in gram positive bacteria. This is the reason gram staining is also known as differential staining [13, 14, 15, 16].

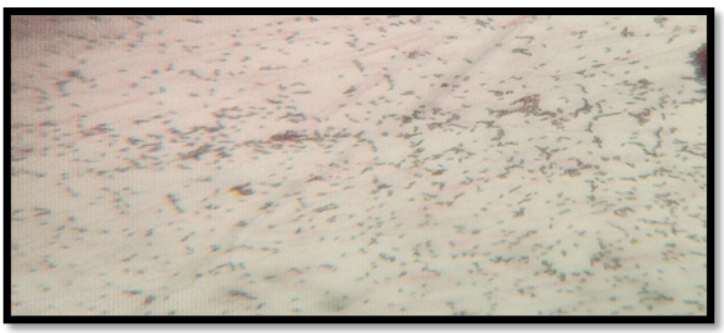


Figure 3: Gram's staining, the morphology of isolated showed Gram-negative, pink colored, medium rod-shaped appearance

Biochemical Tests

Biochemical test was performed to know the chemical nature of the specimens through which the results were made. The biochemical includes catalase test, TSI, Oxidase, nitrate reduction, starch hydrolysis etc. for the biochemical tests several different types of reagents were used which reacts with the metabolic activity of the bacteria and results were observed. All the test media were first measured according to the manufactures detailed then autoclaved at 121 for 15 mints at 15 psi. then it was allowed to cool, once the media were cooled down the selected inoculum were transferred from the freshly made cultures then labelling was done according to requirement. Once the inoculation was done in the respective test tubes or petri discs they were incubated to the incubator at 37 for 24 – 48 hrs. time depends on the criteria of the tests. Once the incubation period was completed the reagents were added to the test tubes and observed for the result. It is always important to take care about the contamination related precautions [17, 18, 19, 20, 21, 22].

Urease test: This test was performed to determine the production of urease enzyme by the microorganism; this test was performed in the test tube in which urease enzyme attack the carbon and nitrogen bond amide with the liberation of the ammonia. During the incubation period the isolates reduces ammonia which increase the pH of the media due to which phenol red changes from yellow color to the red color or pink color, which indicates the positive result and no changes color of the media shows the negative result [17, 18, 19, 20, 21, 22].

Methyl Red test: This test determines the ability of microorganism to fermenting with the production of acid as end products. The isolated microorganisms were inoculated in Methyl Red-Voges Proskauer broth. All the inoculated were incubated at 37 for 24 hrs. After incubation, 5-6 drops of methyl red reagent were added to the broth/media. A Red color of medium indicates positive test, while no color change shows negative result [17, 18, 19, 20, 21, 22].

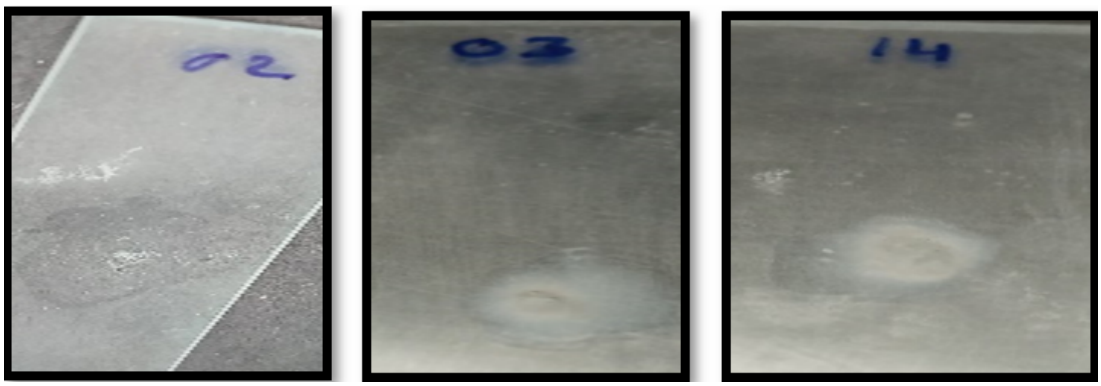
Voges Proskauer Test: This test determines the capability of microorganism to produce non-acidic end products such as ethanol and acetoin from the organic acid. The isolated microorganisms were inoculated in Methyl Red Voges Proskauer broth. All the inoculated were incubated at 37C for 24 hrs. After incubation, 12 drops of freshly prepared VP-reagent I (naphthol solution), 2-3 drops of VP reagent II (40% KOH) were added in all the inoculated and in controls. Development of crimson to pink (red) color indicated positive test no change in color indicated negative test [17, 18, 19, 20, 21, 22].

Indole Test: This test determines the ability of the isolate to hydrolyze tryptophan with the production of indole and pyruvic acid by the production of tryptophanase enzyme. The isolated organism was inoculated in tryptone broth. All the isolates were inoculated and incubated at 37 for 48 hrs. After incubation a reagent was used, added 5 drops of Kovac's reagent into the media Cherry red color appears at the top layer which indicates positive result no color changes show negative result [17, 18, 19, 20, 21, 22].

Casein Hydrolysis Test: This test determines the ability of isolates to break down milk protein casein by the activity of caseinase enzyme which hydrolyzed casein into small amino acid. Skimmed milk agar was used for this test, the test isolates were incubated into the media and incubated at 37 for 24 hrs. clear zone shows positive result and no clear zone negative result [17, 18, 19, 20, 21, 22].

Gelatin Hydrolysis Test: This test was performed to determine the production of enzyme gelatinase which break down gelatin. Nutrient gelatine broth was used to inoculate the isolates and incubated at 37 for 24hrs or 48 hrs. and after 48 hrs. all the tubes were placed in refrigerator for 2 hrs. gelatine was still liquid shows positive test, as it hard shows negative result. This test is also known as liquefaction [17, 18, 19, 20, 21, 22].

Nitrate reducing test: Trypticase Nitrate broth was used for this test. Isolates were inoculated in this media at 37 for 24 hrs. Two reagents were used α-naphthylamine and sulfanilic acid were added to the media, these both compounds react with nitrite and turn red in color. The tubes were turn red because α-naphthylamine and sulfanilic acid are already present in the tube. In few tubes nitrate was further reduced to ammonia or nitrogen gas. To distinguish between these two reactions, zinc dust was added. Zinc reduces nitrate to nitrite. The test organisms were able to reduce nitrate. Bright red color after the addition of α-naphthylamine and sulfanilic and no color change upon the addition of zinc was recorded as positive nitrate reduction test [17, 18, 19, 20, 21, 22].



A **B** **C**

Figure 4: Catalase test

All the isolates show the positive result as bubble formation occurred after a drop of hydrogen peroxide.

Figure 5: Methyl red test

Four isolates show positive result (red) and one isolate show negative result (yellow).

Figure 6: Urease Test

Isolates show positive result (pink) due to urease production

Table 2: Isolates show following biochemical test results

Sl. No.	Samples	Catalase Test	Starch Hydrolysis	TSI Test	Oxidase	Urease	M. P	VP	Indole	Casein	Gelatine	Nitrate Reducing Test	Citrate
1	SHI-1	+ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve
2	SHI-2	+ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	-ve
3	SHI-3	+ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve
4	SHI-4	+ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve
5	SHI-5	+ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	-ve

Effect of Temperature

Temperature affects intracellular c-di-GMP level. c-di-GMP mediates the production of factors required for biofilm formation. Because the transcription of cdrA is up- or downregulated according to the c-di-GMP level in P. aeruginosa, it can reflect the intracellular c-di-GMP level. Therefore, we measured the expression of the cdrAp- lacZ reporter with temperature to know the intracellular c-di-GMP levels. Our result showed that it decreased rapidly as the temperature rose from 20 to 25°C, and there was no significant change above 25°C.

The reason why biofilm formation increased little by little above 25°C without a significant change in c-di-GMP levels is not clear, but we note that Townsley and Yildiz reported that c-di-GMP levels increased slightly but significantly as the temperature rose from 25 to 37°C in P. aeruginosa PAO1.The expression of alg and pel genes is significantly increased at 20°C. In P. aeruginosa, c-di-GMP controls biofilm formation by modulating the production of alginate, Pel, and Psl, the major EPSs. To assess whether temperature influences the expression of the operons encoding these EPSs, the transcriptions of alg, pel, and psl were measured by using their promoter-lacZ fusion reporters. The results showed that the expression levels of pel, alg, and psl operons were also dramatically affected by temperature, but the patterns were different. The expression of alg rapidly decreased from 20 to 25°C, reaching a low point at 25°C, and then slightly increased again, which was the most similar to the actual biofilm formation pattern.

There are three different temperatures were selected for the formation of biofilm in p.aeruginosa, which are as follows.

a. 4

b. 25
c. 45
The results show different impact of temperature on the biofilm formation by P.aeruginosa.

Figure 7: some isolates show heavy to medium biofilm formation at 4

Figure 8: Few isolate shows medium to light biofilm formation

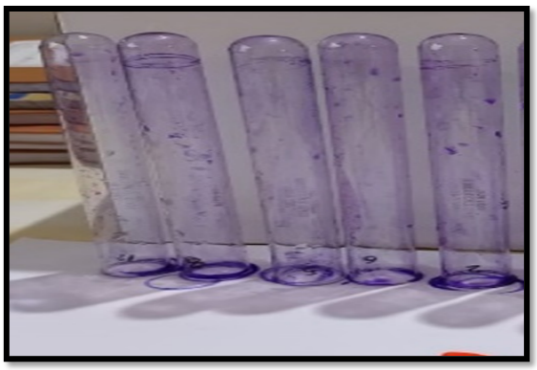


Figure 9: Few isolate shows less biofilm formation

Table 3: Effect of different temperature on the formation of biofilm by P. aeruginosa Ring Formation at different temperature

		Ring Formation at different temperature		
Sl. No	**Isolates**	**Temperature (45°C)**	**Temperature (25 °C)**	**Temperature (4°C)**
1	**SHI-1**	+	++	++
2	**SHI-2**	++	+++	+
3	**SHI-3**	+++	++	++
4	**SHI-4**	+	+	++
5	**SHI-5**	+	+++	+++

+++ = Dark ring, ++ = Light Ring, + = Fade Ring, 0 = no ring
Note: Ring = biofilm formation by the isolates.

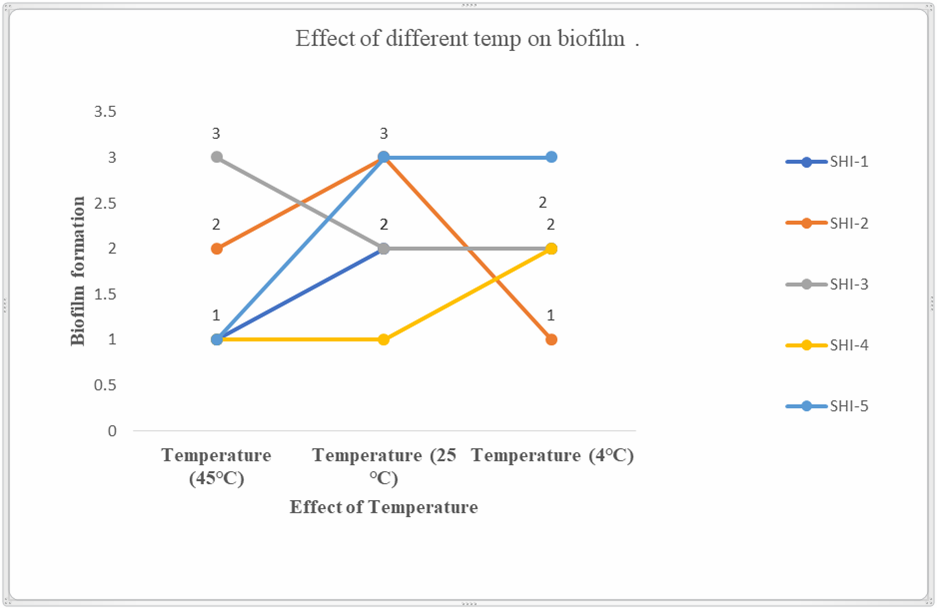


Figure 10: Shows the effect of different Temperature on the biofilm formation by the P. aeruginosa

III. Result And Discussion

The findings from this investigation reveal that *Pseudomonas aeruginosa* exhibits remarkable adaptability when exposed to different temperature conditions. Biofilm formation was detected at all three tested ranges (4°C, 25°C, and 45°C), although the intensity and organization of the biofilm varied.

At low temperature (4°C)**,** growth was initially suppressed and biofilm formation appeared weak, as indicated by faint ring development. With extended incubation, however, the isolates gradually resumed biofilm production, suggesting that *P. aeruginosa* can adjust to cold stress by slowing its metabolism and later activating survival mechanisms. Such a response may explain why the bacterium persists in cooler environments, including hospital drains and storage areas, where it can remain viable despite unfavorable conditions.

At ambient temperature (25°C)**,** biofilm formation was most prominent. The isolates produced thick and well-defined rings, pointing toward active secretion of extracellular polymeric substances (EPS) and optimal bacterial metabolism. This observation supports the idea that moderate environmental conditions promote strong biofilm development, which aligns with previous reports describing the role of EPS in providing structural stability and nutrient retention within bacterial communities.

At high temperature (45°C)**,** biofilm production declined, with only faint rings visible in most isolates. The reduced formation may be attributed to thermal stress, which affects protein stability and overall cellular function. However, the persistence of even a minimal biofilm at this elevated temperature indicates that *P. aeruginosa* activates protective mechanisms, such as heat shock proteins and stress-responsive regulatory pathways, enabling survival in conditions that would normally be inhibitory for many other microorganisms.

A notable outcome of this work is the variation observed among individual isolates. For example, SHI-3 and SHI-5 consistently showed higher biofilm activity across all tested temperatures, while SHI-1 and SHI-4 produced comparatively weaker responses. These differences may reflect genetic variability within the bacterial population, influencing the efficiency of quorum sensing systems, EPS composition, and other regulatory mechanisms. Such diversity highlights the complexity of *P. aeruginosa* as a pathogen and partly explains its ability to adapt to diverse environments. Overall, the results confirm that temperature exerts a strong influence on biofilm formation in *P. aeruginosa*. The organism does not completely lose its ability to produce biofilms at either low or high extremes, but rather modifies the intensity and organization of the matrix. This adaptive capacity provides a survival advantage and contributes to its persistence in clinical and environmental settings. Since biofilms are closely linked to antimicrobial resistance, understanding how environmental factors shape their development is essential for designing better strategies to control *P. aeruginosa* infections.

IV. Conclusion

It was concluded that the p. aeruginosa is one of the adoptive pathogens which causes so many infections in the all-age groups. It was concluded from the experiments that it has a quality of adaption to their surrounding and its genetic make-up makes it more rough and tough toward adverse conditions. It was observed that after drastic changes in the temperature from lower to higher temperature still the p. aeruginosa was able to grow lower as 4 and higher as 45 when the incubation time was exceeded from 24 hrs. to 72 hrs. there

adaptive and genetic orientation helped to form the most important biofilm formation as seen in the experiment by the ring formation at the bottom of test tubes after the addition of crystal violet , a purple color ring as formed round the bottom of tubes. So it was concluded that the p. aeruginosa has survivable capacity which helps it to show pathogenicity and virulence factors in it.

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