Section: Research Paper

Identification of *Pseudomonas aeruginosa* from raw milk and colony counting of *pseudomonas aeruginosa* in nutrient agar medium containing sodium chloride

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Abstract

In the present study, raw milk sample was collected from dairy farms to isolation and identification of *Pseudomonas aeruginosa*. The identification of *P. aeruginosa* was carried out using gram staining, capsule staining and endospore staining. Inhibition of *P. aeruginosa* was also determined by colony counting method up to 10 days after inoculation on nutrient agar medium supplemented with sodium chloride that used as anti-bacterial agent. In gram staining, pink color of gram-negative bacteria was observed and in capsule staining, bacterial cells and the proteinaceous background appeared purplish while the capsules appeared transparent. Endospore staining of *P. aeruginosa* was also positive with visualized endospores in contrast to vegetative cells. All the three staining confirmed the *P. aeruginosa* bacteria. Whereas the result of anti-bacterial method revealed that the growth of *P. aeruginosa* was completely inhibited at all observation days due to the sodium chloride presence in nutrient agar medium. The present research depicts the significance of NaCl for aseptic purposes and is one of the best natural harmless antiseptic which has major prospects in general public health measures.

Keywords: *Pseudomonas aeruginosa*, Gram staining, Capsule staining, Endospore staining, Sodium chloride, Colony counting, Anti-bacterial agent.

Introduction

To ensure the safety and quality of livestock products, preventing microbial spoilage is of utmost importance (Zhang *et al.*, 2014). This requires a comprehensive understanding of various factors, such as potential hazards, their likelihood of occurrence in different products, their physiological characteristics, and the effectiveness of preventative measures (Blackburn, 2006). Milk, which is a highly nutritious food of animal origin, provides an ideal environment for microorganisms to

thrive. Hence, it is crucial to maintain quality control of milk and milk products to reduce the risk of food poisoning outbreaks (Bashir *et al*., 2014).

After the implementation of refrigerated storage for raw and processed foods, psychotropic bacteria have become a major problem for the food industry. Among these bacteria, *Pseudomonas* species are the most prominent Gram-negative rods found in refrigerated foods, making up approximately 50% of the total bacteria present. *Pseudomonas aeruginosa*, in particular, has been identified as the primary cause of inflammatory infections and various systemic infections, including those affecting the urinary, respiratory, and gastrointestinal tracts, as well as soft tissue, bone, and joint infections. This pathogen is frequently found in farm environments, particularly in water sources and contaminated water used for udder washing, and is a common cause of pseudomonas mastitis in cows. Due to its ubiquitous presence in water systems and ability to acquire antibiotic resistance, *P. aeruginosa* is an especially dangerous and concerning pathogen. In addition to causing economic losses by reducing the quality of hides, this bacterium can also contaminate milk, predispose cows to mastitis, and cause infections in immunocompromised individuals who consume contaminated milk (Swetha *et al.*, 2017).

The reduction of pathogens and spoilage microorganisms could be achieved through the development of new, non-toxic, food-compatible chemical products (Cabezas-Pizarro *et al.* 2018). Previous studies have shown that short-chain organic acids and their salts can inhibit the growth of bacteria and molds, including pathogenic strains (Wang *et al.*, 2000; Zainab *et al.*, 2011).

However, limited research has been conducted on the isolation of *P. aeruginosa* from milk samples, and the available data are inconsistent (Swetha *et al*., 2017). Furthermore, no detailed investigations have been carried out on the antibacterial properties of sodium chloride. Given the severity of *P. aeruginosa* infections and their impact on human health, this study aimed to isolate *P. aeruginosa* from raw milk and assess the potential of sodium chloride to inhibit its growth.

Material and methods

A. Sample collection

Milk samples were collected for isolation and identification of *Pseudomonas aeruginosa* from organized dairy farms, Jaipur, Rajasthan, India. Milk sample of 25 ml was collected aseptically from animal in a sterile screw capped bottle and immediately, were kept in ice box in which temperature was maintained at 40°C ± 10°C.

B. Isolation and Identification of *Pseudomonas aeruginosa*

Serial dilution method was used to isolate the separate colony of *Pseudomonas aeruginosa*. 10^{-5} serially diluted milk sample was inoculated on nutrient agar medium (Himedia Pvt. Ltd, India)

and then incubated at 30°C for 24 h. The colonies were touched by inoculation loop and smears were prepared further followed by gram staining.

i) Gram staining

In the Gram stain, the cells are first heat fixed and then stained with a basic dye, crystal violet. The slides are then treated with an I_2-KI mixture (mordant) to fix the stain, washed briefly with 95% alcohol (de-stained), and finally counterstained with a paler dye of safranin. Gram-positive organisms retain the initial violet stain, while gram-negative organisms are decolorized by the organic solvent and hence show the pink counterstain (Hildbrand and Scroth, 1972).

ii) Capsule Staining

First a smear was prepared from the isolated slant culture grown nutrient agar medium and allowed to air dry. The slide was covered with 1% crystal violet for 2 minutes. This was further rinsed gently with a 20% solution of copper sulfate and then dried it. Examination of the slide was carried out under an oil immersion lens.

iii) Endospore staining

A bacterial smear was taken on a clean slide, air dried and gently heat fixed. Then the slides were flooded with malachite green, for 3-5min using the flame of burner. The slides were washed gently in flow of tap water to remove dye. After cooling the slides, safranin was drained on to the slide. The slide was washed gently in flow of tap water and air dried. The slides were observed at 100X with oil immersion and data was recorded for different isolates. Ultrastructure of isolated bacteria was observed through florescent microscope to confirm the final bacterial identification.

C. Colony counting

After the complete verification of the bacterium, the colony counting was carried out. The cell count of isolated bacteria was done by hemocytometer following Andersen and Throndsen, 2004 method. The hemocytometer chamber was filled with spirulina culture of each media (Zarrouk's media, Zarrouk's modified media, BG-11 media and F-2 media) by capillary action. The whole slide or a selected number of large squares was observed under microscope for counting the significant cell number. The cell count was carried out from 1^{st} day after streaking up to 10^{th} day. The average cell number in one ml sample was calculated by using following formula:

The average number of cells per ml = average count per large square X 10,000

D. Antibacterial effect of sodium chloride

1 gm of sodium chloride was added to 20 ml of nutrient agar (NA) medium. Streaking of isolated bacteria was done on solidified NA plates. These were incubated for 24 h at 30°C. Experiment was repeated in triplicate forms. The average colony count in 3 replicas is noted. The cell count was carried out on NA medium supplement with NaCl from 1^{st} day after streaking up to 10^{th} day.

E. Statistical analysis

In triplicate forms all the experiments were done and the results were represented as mean ± standard error [SE].

$$SE = SD / \sqrt{N}$$

Here, SD denotes standard deviation and N denotes number of observations or sample sizes

Result and Discussion

Identification of *Pseudomonas aeruginosa*

The colonies on NA plate were found to be white to pale in color and round to oval in shape. Gram staining showed the *P. aeruginosa* cells to be pink and hence gram negative whereas other bacteria in consortium by milk isolation were gram positive. It was rod shaped and the measurement by micrometer was about 1-5 µm long and 0.5-1.0 µm wide shown in Figure 1. In the present study focused on gram negative *P. aeruginosa*. In capsule staining, bacterial cells and the proteinaceous background appeared purplish while the capsules appeared transparent. Other bacteria in the consortium were non-capsulated and hence this showed further proof of *Pseudomonas aeruginosa* bacteria (Figure 2).

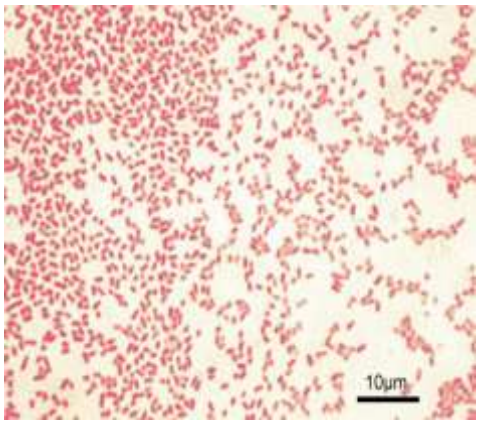

Fig. 1: Gram staining of *P. aeruginosa*

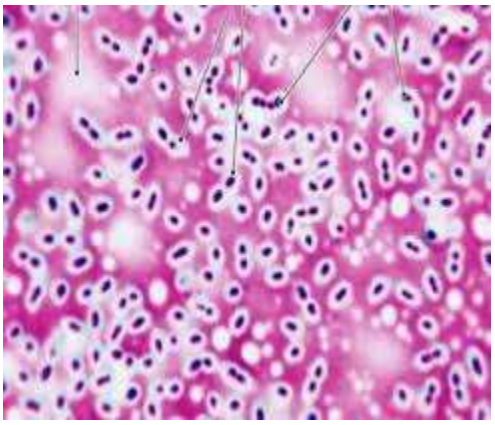

Fig. 2: Capsule staining of *P. aeruginosa*

Endospore staining shown in Figure 3 of *P. aeruginosa* was positive, as this bacterium has the capability of endospore formation for perennation. The endospores were visible and appeared in contrast to vegetative cells. Ultramicroscopically, *P. aeruginosa* showed a single and supercoiled circular chromosome in the cytoplasm. It also showed a lot of chromosome-mobilizing plasmids that are very significant to the organism's lifestyle as a pathogen.

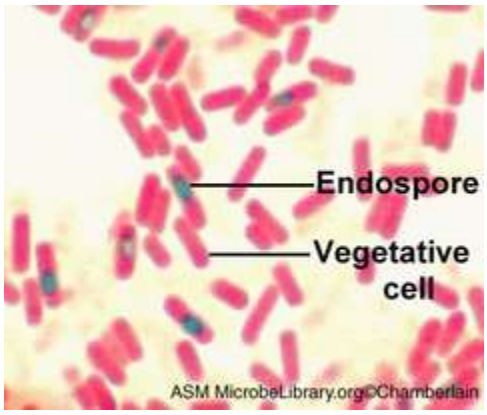

Fig. 3: Endospore staining of *P. aeruginosa*

All the three staining processes utilized for confirming *P. aeruginosa*, gave its identification and characteristics different from other gram positive bacteria in diluted milk sample consortium. Likewise in 2022, Du *et al.* isolated a total of 116 Pseudomonas species such as *P. fluorescens, P. extremorientalis, P. rhizosphaerae, P. psychrophila, P. rhodesiae, P. lundensi, P. lactis, P. granadensis, P. veronii, P. lurida* and *P. azotoformans* from 25 raw cow milk samples. Sequence analysis of Azemin *et al.*, 2022 study confirmed the presence of *P. aeruginosa* isolated from Dorper sheep milk's. Swetha *et al.*, 2017 isolated *Pseudomonas aeruginosa* from raw milk collected from local vendors, private dairy farms in and around Tirupati and identification was done by using biochemical tests including cetrimide test, catalase test, citrate utilization test, methyl red test, indole test, voges proskauer test, and triple sugar iron test. They concluded that the prevalence of *P. aeruginosa* in the raw milk may be due to contamination of milk with polluted water

Antibacterial effect of sodium chloride

On the basis of colony counting, anti-bacterial effect of sodium chloride was observed. On NA with sodium chloride, the medium composition converted to crystalline form. Granulated crystals appeared and medium coloration became bright yellow after growth of *P. aeruginosa*. On the first day after inoculation of *P. aeruginosa*, the average colony count in simple NA medium was 120 and on the second day, the number of bacterial colonies was increased i.e. 140 colonies. No bacterial count was seen on NA with sodium chloride on 1st and 2nd days which was estimated to be a good antibacterial. It was concomitantly observed that the bacterial colonies of *P. aeruginosa* started merging after 2 days and the colony count would not be exact if taken. Therefore, it was relevant to rather take approximate percent cover by *P. aeruginosa* on the medium containing simple NA and NA+ sodium chloride. The complete petri-plate was taken to be 100% cover, its quadrant as 25% cover and hemisphere as 50%; the approximate percent cover area was observed and recorded. On the third day colonies merged and covered some area of petri-plate which was found to be 28.9% on an average in simple NA. On the 4th, 5th, 6th, 7th, 8th, 9th and final 10th observation days *P. aeruginosa* covered 50%, 66.66%, 75%, 80%, 93.33%,

96.66%, and 100% area on NA medium described in Table 1. Furthermore, at the all observation days, sodium chloride containing NA medium showed no bacterial growth. This showed that bacteria are not efficient to grow in a good percent of sodium chloride and thus NaCl proved to be the best anti-bacterial agent. However, two other compounds i.e. tri sodium citrate and sodium lauryl sulphate was also evaluated as good sanitizer, but no better result was found as compared to the NaCl. Therefore the result of NA with NaCl was reported in this paper. Figure 4 is shown maximum colony count of *P. aeruginosa* on simple NA medium. Kovanda *et al.,* 2019 exhibited organic acids and their derivatives as significant anti-bacterial agent against gram positive and gram negative anti-bacterial drugs resistant bacteria. The results of Wijnker *et al.,* 2009 reported that sodium chloride has good anti-bacterial potential therefore it is used for the preservation of natural casings. Nagaoka *et al.,* 2010 revealed inhibitory activity of sodium citrate against numerous oral bacteria and *Streptococcus pneumoniae*. Torğut *et al.,* 2020 described the sodium acrylate copolymers as anti-bacterial agent against *S. aureus, S. cohnii, B. megaterium, B. subtilis, E. coli, K. pneumoniae* and *P. aeruginosa* by using disc diffusion method. It was observed that copolymers have significant potential to control the several diseases. In 2018, Cabezas-Pizarro *et al.* reported the concentration levels i.e. 100>50>25mg/ml of aliphatic acid salts to inhibit the pathogen. On the basis of chemical nature, the level of inhibitory effect was decanoic<octanoic <hexanoic<butanoic acid salts. In their study, sodium salts showed good anti-bacterial properties as compared to the potassium salts. However, very little work has been reported on sodium chloride as antibacterial agent. The result of the current study revealed that sodium chloride created halophilic environment in NA medium and hence no growth of *P. aeruginosa* was observed highly saline conditions and rather exosmosis and plasmolysis of its cytoplasm would have destroyed the inoculated bacteria. This hence proved that sodium chloride was the best potential sterilizing agent as compared to tri sodium citrate and sodium lauryl sulphate.

Fig. 4: Simple NA showing maximum colony count of *P. aeruginosa*

Table 1: Colony count in NA medium and NA with Sodium Chloride up to 10th day after inoculation

Days	Medium and chemical Constituent used for sterilization	Colony count in Replica 1	Colony count in Replica 2	Colony count in Replica 3	Average Colony count
1st	Simple Nutrient Agar medium	100	120	140	120
	NA+ Sodium Chloride	Nil	Nil	Nil	Nil
2nd	Simple Nutrient Agar medium	140	140	140	140
	NA+ Sodium Chloride	Nil	Nil	Nil	Nil
3rd	Simple Nutrient Agar medium	25%	25%	30%	28.9%
	NA+ Sodium Chloride	No growth	No growth	No growth	No growth
4th	Simple Nutrient Agar medium	50%	50%	50%	50%
	NA+ Sodium Chloride	No growth	No growth	No growth	No growth
5th	Simple Nutrient Agar medium	70%	60%	70%	66.66%
	NA+ Sodium Chloride	No growth	No growth	No growth	No growth
6th	Simple Nutrient Agar medium	75%	70%	80%	75%
	NA+ Sodium Chloride	No growth	No growth	No growth	No growth
7th	Simple Nutrient Agar medium	80%	80%	80%	80%
	NA+ Sodium Chloride	No growth	No growth	No growth	No growth
8th	Simple Nutrient Agar medium	90%	95%	95%	93.33%
	NA+ Sodium Chloride	No growth	No growth	No growth	No growth
9th	Simple Nutrient Agar medium	95%	100%	95%	96.66%
	NA+ Sodium Chloride	No growth	No growth	No growth	No growth
10th	Simple Nutrient Agar medium	100%	100%	100%	100%
	NA+ Sodium Chloride	No growth	No growth	No growth	No growth

Conclusion

Sodium chloride is best utilized for wound healing, saline gargles, cleansers, added in shampoos, added in mouthwashes, added in toothpastes etc. It also holds important place in case of internal body infections like sore throat, post-operative therapies, stomach problems, respiratory infections etc. In the present study, sodium chloride was proved as anti-bacterial agent due to observe no *P. aeruginosa* colonies on NA medium supplemented with NaCl. *P. aeruginosa* was isolated from raw milk and their identification was confirmed by obtain positive result of gram staining, capsule and endospore staining. Milk is a significant food of human nutrition and the growth of pathogen, therefore the control of microbial spoilage in milk and milk products is crucial. Here sodium chloride was showed significant inhibiting agent to pathogen. Hence, sodium chloride can be used as best sanitizers to safe human health.

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