

Detection of *Proteus* spp. in Artificial Surface Samples and Estimation of the LOD of the Qualitative Microbiological Method [†]

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Abstract

Food contact surfaces can be a source of food contamination. Bacteria of the genus *Proteus* are known as opportunistic pathogens, often associated with faecal contamination and decomposition of organic matter. This study aimed to isolate *Proteus* spp. from surface samples (of dimensions 5 cm² × 5 cm²). Three levels of artificially soiled aluminium foil were prepared using bacterial suspensions of *Proteus hauseri* ATCC 13315. Afterwards, the surface swabbing method for the detection of *Proteus* spp. was applied. The swab was homogenised with Eugon LT 100 broth, and 1 mL was transferred to the enrichment broth. After the incubation of the enrichment broth, streaking on the Brilliant Green Agar and Salmonella Shigella Agar was performed. The characteristic colonies were confirmed by biochemical reactions. The number of positive findings of *Proteus hauseri* on the applied level of contamination was used for calculation by the PODLOD_ver12.xls ECEL program by Wilrich and Wilrich. This program estimates the probability of detection (POD) function and the limit of detection (LOD) of qualitative microbiological methods. The results of the detection of *Proteus hauseri* in surface samples showed LOD₅₀ = 24.60 [48.96; 97.45] CFU in 1 mL of swab rinse, and LOD₉₅ = 106.30 [211.59; 421.15] CFU in 1 mL of swab rinse. The applied method for isolation of *Proteus* spp. from the surface samples can be used for well-contaminated surfaces.

Keywords: *Proteus* spp.; limit of detection; surface contamination; swab sampling



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1. Introduction

Bacteria of the genus *Proteus* indicate faecal contamination of water or soil, and may play an important role in the decomposition of organic material [1]. *Proteus* bacteria are known as opportunistic pathogens in human and animals that can cause a variety of infections and are often isolated from the intestines of clinically healthy humans and animals without symptoms of disease [2]. Kozlovskaya [3] reported that *Proteus* bacteria-associated pathology in humans and animals has increased significantly in recent years with intestinal, respiratory, and urinary tract diseases and involvement in the development

of kidney and bladder stones. For example, bacteria from *Proteus* species have been isolated in 70% of cases of bacterial urinary stones [4].

Bacteria of the genus *Proteus* are responsible for food spoilage. Of the 60 tested samples of minced meat from the Alkarkh area of Bagdad Province, Iraq, 5 samples (8.33%) were positive for *Proteus* spp. [5]. Yu et al. [6] isolated *P. mirabilis* from 29 out of 80 broiler carcasses with a mean contamination level of 2.25 ± 0.50 lg CFU/g. Wang et al. [7] reported that *P. mirabilis* was responsible for a case of food poisoning that occurred in August 2008 in a group of 13 individuals who consumed stewed pork balls in brown sauce in Beijing. Also, there are growing concerns about antimicrobial resistance and the transfer of antimicrobial resistance genes in the environment. Ma et al. [8] isolated 89 *P. mirabilis* strains from 347 samples of retail meat products in food markets across China and reported that high contamination of *P. mirabilis* can serve as a source for various clinically important antimicrobial resistance genes. Sanches et al. [9] tested the antimicrobial resistance of 32 *P. mirabilis* strains isolated from chicken carcasses for antimicrobial resistance and discovered that 25 (78.13%) of the strains were multidrug-resistant. Handling of contaminated meat poses a risk of surface contamination and health hazards.

The presence of *Proteus* spp. on the surface of food processing equipment indicates inadequate sanitation and hygiene practices [10]. Microbiological contamination of surfaces in food production facilities and food-handling areas challenges the prevention of cross-contamination [11]. Therefore, assessing the microbiological purity of surfaces that come into contact with food necessitates a rigorous methodological approach. The objective of the study was to design a method to determine the limit of detection of *Proteus* spp. from artificially contaminated surface samples.

2. Materials and Methods

2.1. Artificial Contamination of Surfaces

The prepared bacterial suspension with a density of 0.5 McF was decimally diluted, and 1 mL of dilutions 10^{-6} and 10^{-7} was transferred to a Petri plate in duplicate and poured with Plate Count Agar (PCA) (HiMedia, Maharashtra, India) following ISO 4833-1 [12]. The Petri plates were incubated at 37 °C for 72 h. After incubation, the number of cells of *P. hauseri* in suspension was counted to estimate the cell count on the contaminated surfaces.

The three levels of artificially contaminated aluminium foil were prepared using dilutions of a suspension of *P. hauseri* ATCC 13315 and spread with Z-shaped rods within a stainless-steel frame (5 cm × 5 cm) for each surface. For the 1st level of contamination, 20 contaminated surfaces (5 cm × 5 cm) spreading 0.1 mL of dilution 10^{-5} were prepared. For the 2nd level of contamination, 10 surfaces spreading 0.25 mL of dilution 10^{-5} were prepared. For the 3rd level of contamination, 5 surfaces spreading 0.2 mL of dilution 10^{-4} of a suspension of *P. hauseri* were prepared. One aluminium foil surface with no artificial contamination (blank control) was also prepared for testing.

2.2. Surface Sampling, Isolation and Identification of *Proteus* spp.

After swabbing the surface with a cotton swab stick following the standardised method ISO 18593 [13], the determination of *Proteus* spp. was performed. The swab was homogenised with 25 mL Eugenic LT 100 broth (HiMedia, India), and 1 mL of broth was transferred to Nutrient broth (Torlak, Belgrade, Serbia). After incubation of the Nutrient broth for 18 ± 2 h at 37 ± 1 °C, streaking on the Brilliant Green Agar (BGA) (HiMedia, India) and Salmonella Shigella (SS) Agar (Oxoid, UK) was performed. BGA and SS plates were incubated at 37 ± 1 °C for 24 ± 2 h. Picked colonies were streaked to Tryptone Soy Agar (TSA) (Oxoid, Basingstoke, UK) and incubated at 37 ± 1 °C for 24 ± 2 h. The colonies from TSA agar were subjected to biochemical reactions and identification by an API test

(bioMérieux, Crappone, France) as well as a haemolysis test on a Blood agar plate (Torlak, Belgrade, Serbia).

2.3. POD and LOD Calculation

The number of positive findings of *P. hauseri* on the applied level of contamination was used for calculation by the adopted PODLOD_ver12.xls ECEL program by Wirlich and Wilrich [14]. This program estimates the probability of detection (POD) function and the limit of detection (LOD) of qualitative microbiological methods. The probability of detection (POD) function, $p(d)$, is the functional relationship between the probability p of obtaining a measurement result 1 and the contamination of the test material, quantitatively expressed as the number of CFUs of the microorganism of the defined type per unit of weight or volume, d (CFU/g or CFU/mL) [15]. The calculation of POD and LOD of detected *Proteus hauseri* from artificially contaminated surfaces was changed in part of the unit, i.e. CFU/cm² instead of CFU/g or CFU/mL.

3. Results and Discussion

The artificially contaminated aluminium foils simplified isolation and detection because the surfaces were contaminated with known monoculture *P. hauseri* ATCC 13315. The isolated colonies show non-swarming on the TSA (Figure 1) and Blood agar plate (Figure 2a) after 24 ± 2 h of incubation. Growth of *P. hauseri* on the Blood agar plates after 26 h of incubation (Figure 2b) showed no haemolysis. After prolonged incubation of plates for five days, alpha haemolysis appeared, indicating mild swarming. Both used media had 5.0 g/L of sodium chloride (NaCl). Nissanka et al. [16] investigated the growth of *P. mirabilis* on Nutrient agar plates prepared with a range of sodium NaCl concentrations from 0.1 to 5.0 g/L. They concluded that *P. mirabilis* did not swarm on modified Nutrient agar with a NaCl concentration of ≤ 0.66 g/L.

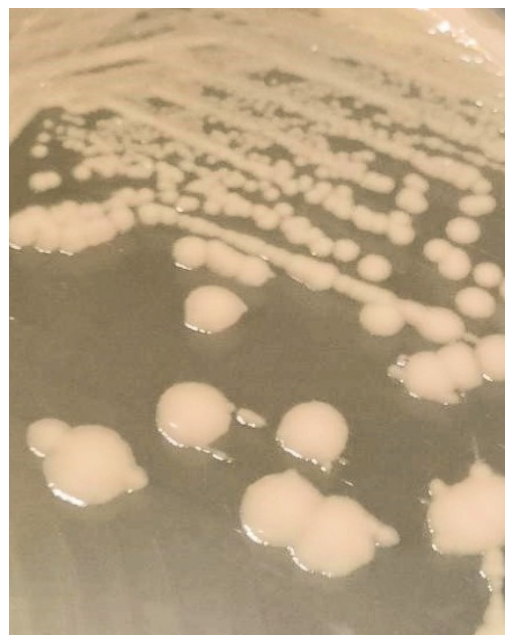


Figure 1. Non-swarming colonies of *P. hauseri* on TSA after 26 h of incubation.

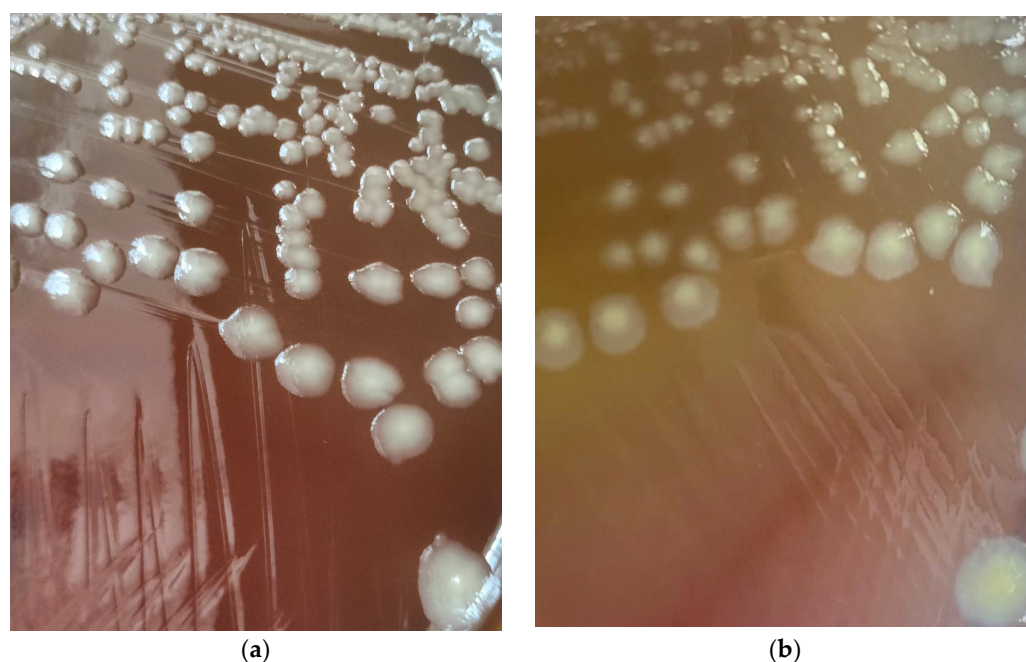


Figure 2. Colonies of *P. hauseri* on the Blood agar plate: (a) after 26 h of incubation; (b) after 5 days of incubation.

Due to its non-swarming nature, many isolates of *P. hauseri* from naturally contaminated samples and mixed cultures can be missed [17]. Swarming is the main micromorphological characteristic of *Proteus* bacteria, which enables the bacteria to migrate on a solid surface and form a biofilm, particularly on the surface of a urinary catheter [18]. Kishore [19] noted that *P. penneri* may be non-swarming on first isolation. Some psychotropic drugs can inhibit swarming in *Proteus*, and swarming appears to be a sign of the pathogenicity of the species [20].

In addition to morphological characteristics, the detection identification of *Proteus* spp. also requires knowledge of the diversity of biochemical characteristics of the members of this genus [21]. The genus *Proteus* also includes *P. alimentorum* sp. nov. [22], *P. appendicitidis* [23], *P. cibi* and *P. faecis* [24], *P. columbae* [25], *P. genom* sp. 4, *P. genom* sp. 6, and many unclassified *Proteus* species. *P. hauseri* can be distinguished from *P. mirabilis*, *P. penneri*, and *P. myxofaciens* as it is positive for indole production, and the other three are negative [17].

The test results of the artificially contaminated surfaces are presented in Table 1. The number of tested surfaces and the number of surfaces with detected *Proteus hauseri* are used for the calculation of POD (Figure 3) and LOD, following Wirlich and Wilrich [15].

Table 1. Results of detecting *Proteus hauseri* from artificially contaminated surfaces.

Level of Surface Artificial Contamination	Estimated Artificial Surface Contamination (CFU/25 cm ²)	Estimated Artificial Surface Contamination (CFU/cm ²)	Number of Tested Surfaces	Number of Surfaces with Detected <i>Proteus hauseri</i>
Blank control	0	0	1	0
I	290	11.6	20	6
II	725	29	10	3
III	5800	232	5	4

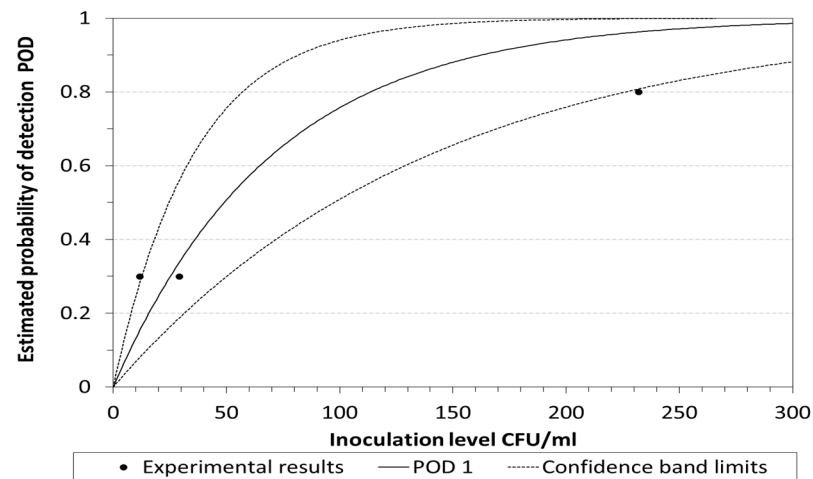


Figure 3. Estimated POD curve of the combined ideal method and its 95% confidence band.

The LOD calculation of results showed $\text{LOD}_{50} = 48.96$ [24.60; 97.45] CFU of *P. hauseri* in 1 mL of swab rinse and $\text{LOD}_{95} = 211.59$ [106.30; 421.15] CFU in 1 mL of swab rinse. The tested 1 mL of swab rinse is equivalent to 1 cm² because the swab sample from 25 cm² was homogenised in 25 mL of Eugenic LT 100 broth, and 1 mL of homogenate was used for enrichment. So, based on applied analysis and calculation, the applied method is less suitable for low-level contaminations. The reasons for such results and the low levels of positive findings can vary and could include many factors. One of the reasons could be the efficiency of the swab stick, depending on the shape of the swab head and its material. Jansson et al. [26] evaluated the sampling efficiencies for 15 different swabs made of cotton (5), flocked nylon (3), and foam (7) and concluded that the recovery varied substantially between swabs made of the same material. The choice of the suitable type of swab will increase the swab efficiency, according to Keeratipibul et al. [27]. Another reason could be the loss of the bacterial suspension on a Z-shaped rod during the spreading on the aluminium foil and, therefore, the absence of quantitative application of the *P. hauseri* suspension. Đurđević-Milošević et al. [28] reported that after sampling from artificially contaminated aluminium foil and comparing the result with the estimated number of *Escherichia coli* in the overnight suspension, the recovery of *Escherichia coli* after swab sampling was less than 60% on PCA. The estimated LOD based on the analysis of an artificially contaminated surface may be lower for naturally contaminated surfaces, due to the loss during the preparation of the contaminated surfaces.

Antibiotic resistance of *Proteus* spp. is a growing trend that is a cause of worry regarding public health [29], especially due to biofilm formation [30]. However, the isolation and identification of *Proteus* spp. are important for monitoring the effects of surface disinfection, preventive hygienic action, and biocontrol.

4. Conclusions

The methods of surface sampling and isolation of microorganisms play a key role in the inspection and control of surface safety in the food industry. Surface swabs were taken from artificially contaminated surfaces with *P. hauseri* ATCC 13315 suspension. The estimated limit of detection of an applied method for isolation of *Proteus* spp. from the surface samples indicates this method can be used on well-contaminated surfaces. Swarming on solid culture media usually indicates the presence of *Proteus* spp. However, the absence of swarming is not a reliable indicator of the absence of *Proteus* spp., and special care should be taken when isolating *Proteus* spp. from mixed cultures, and this should be followed by

biochemical tests for identification. Future work should include more species of the genus *Proteus*, isolation from mixed cultures, and sampling with different types of swab sticks.

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