

ORIGINAL ARTICLE

Bacteriological Profile and In-Vitro Biofilm Formation on Tracheostomy Cannulae at Four Weeks: A Cross-Sectional Study in Padang, Indonesia

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ABSTRACT

Background: A tracheostomy tube bypasses the mucociliary escalator and is colonised rather than kept sterile, yet we identified no published study from Sumatra characterising what grows on it, and none anywhere sampling at a device age fixed by protocol.

Objective: To characterize the cannula bacteriology and to quantify in-vitro biofilm production on tracheostomy tubes at a protocol-fixed device age of four weeks in Padang, Indonesia.

Methods: This cross-sectional study enrolled 33 consecutive adults at a protocol-fixed device age of four weeks at Dr. M. Djamil General Hospital, Universitas Andalas, Padang, West Sumatra, between January and July 2024. The inner aspect of the distal outer cannula was swabbed, cultured on blood agar and identified by MALDI-TOF mass spectrometry; biofilm production was quantified by the tissue-culture-plate assay at 570 nm in triplicate against the plate-specific negative control.

Results: Every specimen yielded growth (33/33; 100%, 95% CI 89.6–100). Gram-negative bacilli accounted for 93.9% (95% CI 80.4–98.3) of isolates and non-fermenters for 60.6% (43.7–75.3); *Pseudomonas aeruginosa* was commonest at 51.5% (35.2–67.5). Moderate or strong biofilm production was found in 69.7% of isolates (52.7–82.6) and any biofilm production in 75.8% (59.0–87.2), median biofilm index 3.01 (IQR 1.71–4.55); all *Klebsiella pneumoniae* and *Proteus mirabilis* isolates produced biofilm, whereas *P. aeruginosa* did so in 70.6%. No patient or care factor predicted biofilm production after Holm adjustment, including inner-cannula cleaning frequency (odds ratio 5.26, 95% CI 0.54–270.0; adjusted p = 0.619).

Conclusion: A predominantly non-fermenting Gram-negative flora with a high biofilm phenotype is established by week four, supporting scheduled tube exchange at that interval and adding local device-microbiology evidence where none existed. Antimicrobial susceptibility testing was not performed and is the priority for the next study.

1. Introduction

Tracheostomy is among the most frequently performed airway procedures in tertiary otorhinolaryngology practice, and the population living with a long-term artificial airway continues to grow as intensive-care survival improves. Every such patient carries an indwelling polymer device that traverses the anterior neck and sits within the trachea, bypassing the nose, the pharynx and the mucociliary escalator that normally clears the lower airway. The anatomical consequence, and the biofilm cascade that follows from it, are summarised in Figure 1. The question is therefore not whether the device becomes colonised but what colonises it, how quickly, and in what physiological state.^{1,2} In Indonesia, where a humid tropical climate favours bacterial persistence on surfaces and where much tracheostomy care after discharge is delivered at home by family carers, that question carries direct operational weight.^{3,4} Dr. M.

Djamil Central General Hospital is the tertiary otorhinolaryngology referral centre for West Sumatra and the teaching hospital of Universitas Andalas, serving a province of several million people, predominantly Minangkabau, across a wide and partly rural catchment; it performs tracheostomy both for upper-airway obstruction and for prolonged endotracheal intubation, and follows those patients into the community.

The microbiological identity of tracheostomy-cannula flora is consistent across published settings and is dominated by Gram-negative organisms. The only systematic review and meta-analysis of this literature, covering 49 studies of endotracheal and tracheostomy tubing, found *Pseudomonas*, *Klebsiella*, *Staphylococcus* and *Acinetobacter* to be the dominant genera, and found *Pseudomonas* significantly more prevalent on tracheostomy than on endotracheal tubes (p < 0.0001).¹ Individual series agree: *P. aeruginosa* has

been the leading isolate in prospective and cross-sectional studies of the tube and the stoma, with *A. baumannii*, *K. pneumoniae*, *Proteus* species, *Escherichia coli* and *Enterobacter* following in varying order and *S. aureus* consistently a minority.⁵⁻⁷ These are not incidental organisms: five of the seven species recovered in the present study lie among the pathogens responsible for most of the global burden of bacterial antimicrobial resistance.^{8,9}

What makes their presence on a device consequential, rather than merely a matter of colonisation, is the physiological state they adopt on it. Organisms attach to the conditioned polymer surface, secrete extracellular polymeric substance, aggregate into micro-colonies and mature into a three-dimensional community from which cells disperse to seed the lower airway and to recolonise a freshly cleaned surface (Figure 1B). Cells within that community tolerate antimicrobial concentrations far above their planktonic minimum inhibitory concentrations, through matrix diffusion limitation, nutrient gradients and persister subpopulations — a reversible phenotypic tolerance that a conventional susceptibility report cannot detect.^{10,11} Species differ in how readily they enter it. *P. aeruginosa* couples quorum-sensing control of exopolysaccharide production to a broad efflux repertoire.^{12,13} *K. pneumoniae* is a notably efficient former on abiotic surfaces, and its biofilm capacity travels with resistance and virulence determinants.¹⁴⁻¹⁶ *A. baumannii* persists on dry abiotic surfaces and forms biofilm on indwelling devices.¹⁷ *P. mirabilis* carries a well-characterised adhesion apparatus.¹⁸ *S. aureus* isolated specifically from tracheostomy tubes reliably carries adhesion and accumulation genes.¹⁹

Where the literature diverges sharply is on how much of this flora actually forms biofilm. Reported prevalence ranges from 57% in a South Indian microtitre-plate series to above 90% in Polish inpatient and outpatient cohorts.^{2,20} Some of that spread is real, reflecting device age, tube material and care practice; much of it is methodological, because the answer depends on the assay chosen, on the negative-control threshold applied, and on whether the tube surface or the recovered isolate is examined.^{21,22} Interlaboratory work has quantified how much of the variation in microtitre-plate biofilm quantification is attributable to run and laboratory rather than to the organism, and crystal violet readings are further known to depend on the phase of biofilm development at the time of reading.^{23,24} Two gaps follow. First, almost no series fixes the device age at sampling, so prevalence estimates aggregate tubes in place for days with tubes in place for months, and the interval at which biofilm becomes established — the interval that should govern tube-exchange scheduling — cannot be read from them.²⁵ Second, the quantitative information the assay generates is routinely discarded: studies report a binary positive or negative result when the assay yields a continuous optical density that the accepted four-tier

rule resolves into graded strata.²⁶ A cohort in which every positive isolate is a weak producer and one in which every positive isolate is a strong producer report an identical prevalence and present entirely different clinical problems.

Indonesia's national response to antimicrobial resistance rests on surveillance and stewardship, and both depend on locally generated data about which organisms occupy which niches.^{27,28} Resistant infection in Indonesian tertiary hospitals carries measurable excess mortality, and national syntheses show both a high resistance burden and marked geographical gaps in the evidence.^{29,30} For tracheostomy cannulae, Indonesian evidence is confined to a randomised trial of chlorhexidine cannula decontamination.³ No study from anywhere in Sumatra has described the bacterial flora or the biofilm phenotype of a tracheostomy cannula; the nearest published Sumatran resistance data concern hospitalised children rather than airway devices.³¹ The empirical assumptions guiding cannula care in West Sumatra therefore rest entirely on data from other countries and other climates. The present study was designed as an interdisciplinary response to that gap: the Department of Otorhinolaryngology–Head and Neck Surgery of Universitas Andalas identified the clinical question and performed the standardised assessment and device sampling; the Department of Clinical Microbiology performed culture, mass-spectrometric identification and biofilm phenotyping; and the Department of Public Health and Community Medicine framed the epidemiological design and conducted the statistical analysis.

Two design decisions distinguish this study from its predecessors and are stated here so that they are not mistaken for incidental detail. The first is that device age was fixed by protocol at four weeks rather than merely observed, so that the resulting prevalence refers to a defined point in the life of the tube and can be used to reason about exchange scheduling; to our knowledge no previous tracheostomy-cannula series has done this. The second is that the full four-tier ordinal classification the assay supports is reported rather than collapsed to a binary result. This study aimed to (1) characterise the bacterial species recovered from tracheostomy cannulae at a fixed device age of four weeks at Dr. M. Djamil General Hospital, Universitas Andalas, Padang; (2) quantify in-vitro biofilm production by those isolates, both as a binary outcome and across the full ordinal classification; and (3) examine whether patient characteristics, the indication for tracheostomy, or the frequency with which the inner cannula is cleaned are associated with biofilm production. A fourth objective — linking the biofilm phenotype to antimicrobial susceptibility — was not attainable with the data collected, and the reasons are stated explicitly so that the boundary of the evidence is visible rather than implied.

2. Methods

Study design and reporting

This was a single-centre, cross-sectional, laboratory-based observational study using prospective consecutive sampling. It is reported in accordance with the STROBE statement for cross-sectional studies.³² The unit of analysis is the bacterial isolate; because one predominant isolate was recorded per patient, the 33 isolates correspond one-to-one to the 33 participants, and no clustering correction is required. No aspect of the design, conduct or reporting was changed after the data were seen, with one exception declared here: the ordinal biofilm classification and the assay-plate sensitivity analyses presented below were specified after primary data collection was complete and are labelled throughout as secondary and exploratory respectively.

Measures taken to limit bias

Five features of the design were adopted prospectively to limit bias. Sampling was consecutive rather than selective, so that no discretion was exercised over which eligible patients entered the study. Device age was fixed by protocol at four weeks, removing the variation in exposure duration that confounds series in which device age is merely observed. All specimens were collected by a single operator at a single anatomical site under consultant supervision, limiting between-operator variation in sampling effort. Every isolate was assayed in triplicate. And the laboratory personnel who performed the culture, identification and biofilm assay took no part in the clinical assessment and had no access to the clinical variables, so the outcome was determined independently of the exposures. Residual sources of bias that the design does not address are set out in the Limitations.

Ethical approval

This study received ethical approval from the Health Research Ethics Committee of RSUP Dr. M. Djamil Padang (Approval No. DP.04.03/D.XVI.XI/265/2024). The protocol was declared ethically appropriate against the seven WHO 2011 standards — social value, scientific value, equitable assessment and benefits, risks, persuasion or exploitation, confidentiality and privacy, and informed consent — with reference to the 2016 CIOMS guidelines. Written informed consent was obtained from every participant before enrolment, after explanation of the procedure, its purpose and its benefits. Participant identity and data were kept confidential, and no participant was exposed to any additional intervention, side effect or complication by taking part. The study was conducted in accordance with the Declaration of Helsinki.

Setting and participants

The study was conducted at the Department of Otorhinolaryngology–Head and Neck Surgery and the Clinical Microbiology Laboratory of RSUP Dr. M. Djamil Central General Hospital, and at the Centre for

Diagnostics and Infectious Disease Research of the Faculty of Medicine, Universitas Andalas, Padang, West Sumatra, Indonesia. Recruitment ran from January 2024 until the target sample size was reached, and laboratory work and analysis were completed by July 2024. The source population comprised all patients whose tracheostomy had been performed by the Department of Otorhinolaryngology–Head and Neck Surgery at that hospital and who remained under its follow-up. The full participant pathway, from the source population to the analysed isolates, is set out in Figure 2A.

Patients were eligible if they consented in writing and if their tracheostomy tube had been in situ for four weeks at the time of sampling. The four-week point was fixed by protocol rather than observed, so that every specimen represents the same device age. No exclusion criteria were specified in the protocol, and in the event no eligible patient was excluded. Sampling was consecutive until the target was reached.

Participants were assessed by history and by physical examination of the neck region, performed by the investigator under the supervision of the consultant in the Laryngo-Pharyngology sub-division of the Department of Otorhinolaryngology–Head and Neck Surgery. What was standardised was the supervision, the sampling site and the timing; the clinical assessment itself was not proforma-driven, and no structured symptom inventory, grading of secretions, assessment of stoma condition or record of granulation tissue was made. The variables recorded on the case-record form were age, sex, the indication for tracheostomy (upper-airway obstruction or prolonged endotracheal intubation) and the frequency with which the patient or family carer cleaned the inner cannula (twice daily, or three or more times daily); all are reported in Table 1. The form did not capture comorbidity, immune status, prior or current antibiotic exposure, tube brand, size or material, duration of hospitalisation, or residence, and it did not distinguish patients who remained inpatients at week four from those discharged to home care. These variables are therefore unavailable, and their absence is treated as a limitation rather than filled by assumption.

The four-week sampling point was chosen for three reasons: it coincides with the routine post-tracheostomy outpatient review interval at this centre, so that participants were seen in any event; measurable material wear and surface roughening of polymeric tracheostomy tubes develop over the first month, and surface roughness promotes bacterial adhesion; and four weeks is the lower bound of the exchange window discussed in the clinical literature, so sampling at that point tests the microbiological case for the earlier end of that window.²⁵ The investigator who performed the clinical assessment and collected the specimens was not blinded to the clinical variables; the outcome, however, was a laboratory optical density generated in a different department by personnel not involved in the

clinical assessment, so the scope for assessment bias is correspondingly small.

Sample size

The required sample size was calculated with the single-proportion precision formula

$$n = Z_{\alpha}^2 \times P \times Q / d^2$$

in which $Z_{\alpha} = 1.64$, the assumed biofilm proportion $P = 0.50$ (the value that maximises the required size in the absence of a local estimate), $Q = 1 - P = 0.50$, and the absolute precision $d = 0.15$. This gives $n = 29.9$, rounded to 30; 33 participants were enrolled. The study was therefore designed to estimate a single proportion to a specified precision and not to test any between-group hypothesis, and the associations examined below are secondary to that purpose. Two properties of the calculation should also be stated plainly: it is a precision calculation rather than a hypothesis test, so it confers no power on any between-group comparison; and $Z_{\alpha} = 1.64$ corresponds to a one-sided α of 0.05, equivalent to a two-sided α of 0.10, whereas the analyses reported below use a two-sided α of 0.05. At the observed proportion the achieved half-width of the 95% confidence interval around the primary estimate was 14.1 percentage points, close to the 15-point target.

Otorhinolaryngological assessment and specimen collection

Specimen collection was performed by the investigator under the same consultant supervision, at the fourth week after tube placement and with the tube in situ. A sterile cotton-tipped swab was passed to the inner aspect of the distal end of the outer cannula and rotated against that surface; the site is shown in Figure 1A. This site was chosen deliberately, for three reasons. It is the surface against which the removable inner cannula seats, so it is the surface that patient and carer cleaning is intended to address and on which any failure of that cleaning will be expressed. It is exposed neither to the tidal airflow that scours the lumen nor to the mucociliary clearance of the native airway, so it is the site at which accumulation is least opposed. And published work has shown that the inner surface of the tracheostomy tube carries greater biofilm growth than the outer surface, so sampling there maximises detection sensitivity.² Because a single site was sampled, the study characterises that site and not the whole device; the stoma, the outer surface and the inner cannula itself may carry different flora.⁵

Each swab was inoculated immediately into thioglycollate transport medium and transported to the Clinical Microbiology Laboratory of RSUP Dr. M. Djamil for processing on the same working day; the complete specimen pathway is shown in Figure 2B. One swab was taken per patient.

Swabs were sterile cotton-tipped; the number of rotations and the exact collection-to-processing interval were not recorded, and cotton has lower release efficiency for adherent organisms than flocked

swabs; recovery of adherent cells from the surface is therefore the least controlled step in an otherwise well-specified protocol, and the sampling effort is not fully reproducible from this description. The study's quality-assurance procedure records that specimens were delivered directly to the Clinical Microbiology Laboratory and to the Centre for Diagnostics and Infectious Disease Research and were stored at -80°C , and that all instruments were calibrated by a clinical microbiologist. The protocol does not state at which stage that storage was applied relative to primary culture and to the biofilm assay, so the number of freeze-thaw cycles preceding the assay cannot be established from the record. Freezing generally reduces subsequent recovery and adherence, so if it preceded the assay the most plausible direction of bias is toward under-detection of biofilm production; this is stated in the Limitations rather than resolved by assumption.

Culture, identification and biofilm assay

All microbiological work was performed by the Department of Clinical Microbiology, Faculty of Medicine, Universitas Andalas, on equipment calibrated by a clinical microbiologist. Specimens were incubated at $35\text{--}37^{\circ}\text{C}$ for 18–24 h and subcultured onto blood agar. Colony morphology was examined and Gram stain performed, and species identification was carried out by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry using the Biomérieux VITEK MS system, which is materially more reliable than biochemical panels for non-fermenting Gram-negative bacilli — the group constituting the majority of this cohort.

The manufacturer's default confidence threshold for species-level assignment was applied and no isolate required resolution by an alternative method. One caveat is recorded: some database versions resolve *Acinetobacter baumannii* only to the *A. calcoaceticus*–*A. baumannii* complex, and the species name is used here in that conventional sense.

The predominant colony type, judged by colony count and morphology on the primary plate, was identified and recorded for each specimen; the protocol did not provide for reporting more than one isolate per patient. Culture was qualitative: thioglycollate is an enrichment medium, no quantitative or semi-quantitative colony count was performed, and a heavily colonised cannula and one carrying few organisms are therefore recorded identically as culture-positive.

Biofilm production was quantified at the Centre for Diagnostics and Infectious Disease Research, Faculty of Medicine, Universitas Andalas, by the tissue-culture-plate (microtitre) method, which is the reference phenotypic assay and the only common method that yields a continuous quantitative read-out.^{21,26,33} Each isolate was inoculated into 5 mL of trypticase soy broth and incubated at 37°C for 24 h, then diluted 1:100. Each well of a 96-well flat-bottom polystyrene tissue-culture

plate (Thermo Fisher Scientific) was filled with the diluted culture and incubated at 37 °C for 24 h. Wells were emptied by gentle tapping, washed twice with 0.2 mL of phosphate-buffered saline at pH 7.2, and incubated at 37 °C for 1 h. Adherent biomass was stained with 0.2 mL of 0.1% crystal violet for 10 min; excess stain was removed by two washes with sterile water and the plate air-dried. The bound stain was solubilised in 200 µL of 33% glacial acetic acid, and the optical density was read on an ELISA reader at 570 nm (OD₅₇₀). Every isolate was assayed in triplicate and the mean optical density used.

The negative control was uninoculated trypticase soy broth carried through the identical staining and solubilisation sequence; the number of control wells contributing to each mean and standard deviation was not recorded, and the dispersion across the three replicate wells for each isolate was not retained, so neither the stability of the threshold nor the reproducibility of individual measurements can be quantified from the record. No reference biofilm-producing strain was run as a positive control. These are acknowledged quality-control gaps. A further property of the assay should be noted when the continuous index below is interpreted: crystal violet binding is approximately proportional to adherent biomass only over a limited range and depends on the phase of biofilm development at the time of reading, so the index should not be read as strictly linear in biofilm mass at its upper end.^{23,24}

Classification followed the four-tier rule of Stepanović and colleagues.²⁶ The cut-off value OD_c was defined as $OD_c = \text{mean } OD_{570} \text{ of the negative control} + 3 \times SD \text{ of the negative control}$

and isolates were classified as non-adherent when $OD_{570} \leq OD_c$, weak when $OD_c < OD_{570} \leq 2 \times OD_c$, moderate when $2 \times OD_c < OD_{570} \leq 4 \times OD_c$, and strong when $OD_{570} > 4 \times OD_c$. Two control values were generated over the course of the study: OD_c was 0.203 for the first 17 isolates and 0.225 for the remaining 16. Each isolate was classified against the control value of its own plate, and the influence of this on the results is examined by sensitivity analysis below.

No antimicrobial susceptibility testing was performed. Disc diffusion, minimum-inhibitory-concentration determination, and the CLSI and EUCAST interpretive frameworks formed no part of the protocol, and no antibiotic discs, Mueller-Hinton agar or MIC panels were used. Consequently no susceptibility, resistance, multidrug-resistance, methicillin-resistance or extended-spectrum β-lactamase result is reported anywhere in this manuscript; the standard definitions that a future study would apply are cited in the Discussion for that purpose only.³⁴

Variables and outcomes

The primary outcome was in-vitro biofilm production, defined as an isolate mean OD₅₇₀ exceeding the OD_c of its plate. Secondary outcomes were the four-tier

category and the biofilm index, defined for this analysis as

$\text{biofilm index} = \text{mean } OD_{570} \text{ of the isolate} / OD_c \text{ of its own plate}$

Normalising to the plate-specific control renders isolates read against different control values directly comparable and converts the outcome into a continuous variable on an interpretable scale, in which 1.0 is exactly the detection threshold and 2.0 and 4.0 are the weak-to-moderate and moderate-to-strong boundaries.

One isolate returned a slightly negative optical density and therefore a negative index; this represents measurement noise around a near-zero signal and the value was retained rather than truncated to zero, because truncation would bias the lower tail of the distribution.

Exposure variables were sex, age (analysed continuously and dichotomised at 60 years), the indication for tracheostomy, and inner-cannula cleaning frequency. Two microbiological groupings were examined: *P. aeruginosa* against all other organisms, and non-fermenting Gram-negative bacilli (*P. aeruginosa* and *A. baumannii*) against Enterobacterales and staphylococci.

Statistical analysis

Analyses were designed and performed by the Department of Public Health and Community Medicine, Faculty of Medicine, Universitas Andalas. Distributions were checked with the Shapiro–Wilk test; age ($W = 0.981$, $p = 0.801$) and the biofilm index ($p = 0.453$) were both consistent with normality, but rank-based methods were retained throughout because several subgroups contain fewer than five observations. Continuous variables are summarised as mean ± standard deviation and as median with interquartile range; categorical variables as counts and percentages with 95% Wilson score confidence intervals, and with Clopper–Pearson intervals at the 0% and 100% boundaries where the Wilson interval is not defined.

Because several expected cell counts fall below five, all two-by-two associations were tested with the two-sided Fisher exact test rather than the chi-square test. Effect sizes are reported for every comparison: the conditional maximum-likelihood odds ratio with its exact 95% confidence interval, and Cramér's V. Continuous comparisons used the Mann–Whitney U test with rank-biserial correlation and the Hodges–Lehmann median difference; comparisons across the four species contributing three or more isolates used the Kruskal–Wallis test with epsilon-squared (ϵ^2); and the association between age and the biofilm index used Spearman's rho with a Fisher z-transformed 95% confidence interval, whose coverage at this sample size is approximate. Three comparisons — sex, cleaning frequency and indication — were pre-specified because they are the associations the source protocol set out to test; the remaining six are exploratory. The

Holm step-down procedure was applied separately within each of these two families.

A multivariable model containing sex, cleaning frequency and indication was fitted by Firth penalised logistic regression. Penalisation was chosen in advance of fitting because the minority outcome comprises only eight isolates, giving an events-per-variable ratio of 2.7 against a conventional minimum of ten; under those conditions unpenalised maximum likelihood is biased away from the null. The unpenalised fit, Nagelkerke R^2 and the Hosmer–Lemeshow statistic are reported alongside; these three quantities derive from the unpenalised maximum-likelihood fit and should not be paired with the penalised odds ratios as though they came from one model. The model is presented as exploratory, and no conclusion in this paper rests on it. Two further analyses were added to make the null results and the assay interpretable. First, post-hoc power was computed at the observed proportions and group sizes, and the minimum p-value attainable by any allocation consistent with the observed marginal totals was determined by enumeration, so that a null result caused by an insufficient sample can be distinguished from one caused by a p-value floor imposed by the design. Second, four sensitivity analyses were planned once the two control values were identified: biofilm positivity and the ordinal category distribution were each compared between the two assay plates; the entire classification was repeated with each control value applied to all 33 isolates in turn, giving both a liberal and a conservative reclassification; the plate comparison was repeated within *P. aeruginosa* alone, to separate a plate effect from the differing species composition of the two plates; and the primary outcome was recomputed across a range of candidate thresholds. The observed prevalence was additionally compared with published benchmark values by exact binomial test. Analyses were performed in Python 3.13 with SciPy 1.17 and statsmodels 0.15, the Firth fit implemented directly; two-sided α was set at 0.05.

3. Results

Participants

Thirty-three consecutive adults with a tracheostomy tube in situ for four weeks were enrolled between January and July 2024, against an a-priori minimum of 30. No eligible patient was excluded and no participant had missing outcome data, so all 33 contributed one isolate each to every analysis, as detailed in Figure 2A. The demographic and clinical characteristics of the cohort are presented in Table 1. Twenty-one participants were male (63.6%, 95% CI 46.6–77.8) and 12 female (36.4%, 95% CI 22.2–53.4), a ratio of 1.75 to 1. Mean age was 55.64 ± 12.79 years (95% CI 51.1–60.2), with a median of 56 years (interquartile range 47–66) and a range of 26 to 88 years. The largest age stratum was 50–59 years (12 participants; 36.4%, 95% CI 22.2–53.4), and 10 participants (30.3%, 95% CI

17.4–47.3) were aged 60 years or more. Tracheostomy had been performed for upper-airway obstruction in 19 participants (57.6%, 95% CI 40.8–72.8) and for prolonged endotracheal intubation in 14 (42.4%, 95% CI 27.2–59.2). Twelve participants or their carers cleaned the inner cannula twice daily (36.4%, 95% CI 22.2–53.4) and 21 did so three or more times daily (63.6%, 95% CI 46.6–77.8).

Bacteriological profile

Every specimen yielded bacterial growth: 33 of 33, or 100% (95% CI 89.6–100). Seven species were identified. The complete species distribution, with Gram-stain class and biofilm production for each organism, is given in Table 2 and displayed in Figure 3A. *Pseudomonas aeruginosa* was the commonest, accounting for 17 isolates (51.5%, 95% CI 35.2–67.5), followed by *Klebsiella pneumoniae* and *Proteus mirabilis* with four isolates each (12.1%, 95% CI 4.8–27.3), *Acinetobacter baumannii* with three (9.1%, 95% CI 3.1–23.6), *Staphylococcus aureus* and *Escherichia coli* with two each (6.1%, 95% CI 1.7–19.6), and *Enterobacter cloacae* with one (3.0%, 95% CI 0.5–15.3). Grouped by Gram-stain class, as shown in Figure 3B, 31 isolates were Gram-negative bacilli (93.9%, 95% CI 80.4–98.3) and two were Gram-positive cocci (6.1%, 95% CI 1.7–19.6). Grouped functionally, as shown in Figure 3C, non-fermenting Gram-negative bacilli accounted for 20 isolates (60.6%, 95% CI 43.7–75.3), Enterobacterales for 11 (33.3%, 95% CI 19.8–50.4) and staphylococci for two (6.1%). The corresponding aggregate rows appear at the foot of Table 2.

Biofilm production

Twenty-five of the 33 isolates produced biofilm in vitro: 75.8% (95% CI 59.0–87.2). Eight did not (24.2%, 95% CI 12.8–41.0). The complete quantitative biofilm phenotype is set out in Table 3.

Resolved into the four strata, the picture is more informative than the binary result alone. The biofilm index of every individual isolate, ordered by magnitude and shown against the classification thresholds, is displayed in Figure 4A. Ten isolates were strong producers (30.3%, 95% CI 17.4–47.3), 13 were moderate (39.4%, 95% CI 24.7–56.3), two were weak (6.1%, 95% CI 1.7–19.6) and eight were non-adherent (24.2%, 95% CI 12.8–41.0). Moderate and strong producers together accounted for 23 of 33 isolates (69.7%, 95% CI 52.7–82.6); that is, 69.7% of the flora recovered from a four-week-old cannula was not merely capable of adhering but did so at least twice as densely as the detection threshold. Because culture was qualitative and used an enrichment medium, this proportion is a property of the isolates recovered and is not a measure of biofilm burden on the cannula itself. The median biofilm index across all isolates was 3.01 (interquartile range 1.71–4.55), with a range from –0.12 to 8.67, as recorded in Table 3.

One isolate returned a slightly negative mean optical density and therefore a negative biofilm index; the

value was retained rather than truncated. The closest approach to a threshold in the whole series was an isolate whose mean OD₅₇₀ of 0.224 lay 0.001 units below its plate's control value of 0.225, and it is the only isolate lying within 10% of its threshold.

Biofilm production by species is given in the right-hand columns of Table 2 and displayed in Figure 4B and C. All four *K. pneumoniae* isolates and all four *P. mirabilis* isolates produced biofilm (100%; 95% Clopper–Pearson CI 39.8–100 for each), as did both *S. aureus* isolates and the single *E. cloacae* isolate. Twelve of 17 *P. aeruginosa* isolates were positive (70.6%, 95% CI 46.9–86.7) and two of three *A. baumannii* isolates (66.7%, 95% CI 20.8–93.9). Neither *E. coli* isolate produced biofilm (0%; 95% Clopper–Pearson CI 0–84.2). The highest median biofilm indices were recorded for *K. pneumoniae* (5.08, interquartile range 4.56–5.98) and *P. mirabilis* (4.24, 3.56–5.02), and the single highest value in the study, 8.67, was a *K. pneumoniae* isolate. Across the four species contributing three or more isolates the difference in biofilm index approached but did not reach conventional significance (Kruskal–Wallis $H = 7.59$, $df = 3$, $p = 0.055$; $\epsilon^2 = 0.191$), as annotated in Figure 4C.

Factors associated with biofilm production

No patient, care or microbiological factor was associated with biofilm production. All nine comparisons, with their odds ratios, exact p-values, effect sizes and Holm-adjusted p-values, are presented in Table 4 and displayed as a forest plot in Figure 5A. Biofilm was produced by 17 of 21 isolates from male participants (81.0%, 95% CI 60.0–92.3) and eight of 12 from female participants (66.7%, 95% CI 39.1–86.2); odds ratio 2.07 (95% CI 0.30–14.48), $p = 0.420$, Cramér's $V = 0.160$. Where the inner cannula was cleaned twice daily, 11 of 12 isolates produced biofilm (91.7%, 95% CI 64.6–98.5), against 14 of 21 where it was cleaned three or more times daily (66.7%, 95% CI 45.4–82.8); odds ratio 5.26 (95% CI 0.54–270.0), $p = 0.206$, Cramér's $V = 0.281$. Where tracheostomy had been performed for upper-airway obstruction, 16 of 19 isolates produced biofilm (84.2%, 95% CI 62.4–94.5), against nine of 14 after prolonged intubation (64.3%, 95% CI 38.8–83.7); odds ratio 2.86 (95% CI 0.44–22.97), $p = 0.238$, Cramér's $V = 0.230$. After Holm adjustment across these three pre-specified comparisons, every adjusted p-value was 0.619.

The exploratory comparisons, also listed in Table 4, were likewise null. Age of 60 years or more was not associated with biofilm production (odds ratio 1.40, 95% CI 0.19–17.17; $p = 1.000$), and age analysed continuously showed no correlation with the biofilm index (Spearman $\rho = 0.022$, 95% CI -0.32 to 0.36 ; $p = 0.904$). Neither non-fermenting Gram-negative status (odds ratio 0.43, 95% CI 0.04–3.09; $p = 0.431$) nor *P. aeruginosa* specifically (odds ratio 0.56, 95% CI 0.07–3.66; $p = 0.688$) predicted biofilm production; both point estimates lie below unity, indicating that the dominant organism was, if anything, a slightly less

reliable biofilm former than the minority species. Analysed as a continuous outcome, the biofilm index did not differ by sex ($p = 0.866$), cleaning frequency ($p = 0.750$), indication ($p = 0.841$) or age group ($p = 0.264$), and the ordinal category did not differ by cleaning frequency ($p = 0.580$).

In the exploratory Firth penalised model reported at the foot of Table 4, twice-daily cleaning carried an adjusted odds ratio of 3.63 (95% CI 0.44–29.87; $p = 0.231$), upper-airway obstruction 2.54 (95% CI 0.33–19.78; $p = 0.373$) and male sex 0.69 (95% CI 0.08–6.04; $p = 0.734$).

The odds ratio for male sex is below unity in the adjusted model although the unadjusted estimate was above it; a sign reversal of this kind at eight events across three covariates reflects small-sample instability rather than confounding, and no interpretation is placed on it.

The model explained little (Nagelkerke $R^2 = 0.170$; likelihood-ratio $p = 0.262$) and showed no evidence of poor calibration (Hosmer–Lemeshow $\chi^2 = 2.41$, $df = 2$, $p = 0.300$); at this sample size that test has almost no power, so the absence of evidence of miscalibration is uninformative rather than reassuring. With an events-per-variable ratio of 2.7 the model is presented for completeness only.

The attained power of the three pre-specified comparisons was 33.9%, 26.4% and 15.8% respectively, as shown in Figure 5C. By enumeration over all allocations consistent with the observed marginal totals, the smallest attainable exact p-value was below 0.001 for each comparison, so these null results reflect the effect sizes present and the sample size rather than a floor imposed by the design.

Benchmarking and sensitivity analyses

The observed biofilm prevalence of 75.8% differed significantly from both published poles: from the 57% reported in a comparable microtitre-plate series (exact binomial $p = 0.034$) and from the figure above 90% reported in tracheostomy-tube cohorts ($p = 0.014$). Both comparators differ from the present study in sampling or detection approach, so these tests bound the discrepancy rather than resolve it.^{2,20}

Because two optical-density control values were used across the assay run, biofilm positivity was compared between plates; the comparison is shown in Figure 5B and the corresponding rows appear at the foot of Table 3. Sixteen of 17 isolates on the first plate were positive (94.1%, 95% CI 73.0–99.0) against nine of 16 on the second (56.3%, 95% CI 33.2–76.9); odds ratio 11.53 (95% CI 1.18–594.9), $p = 0.017$, Cramér's $V = 0.442$. This is the largest association observed anywhere in the dataset, and it is a feature of the assay rather than of the patients. The raw optical densities did not differ significantly between plates (Mann–Whitney $p = 0.201$), nor did the normalised biofilm index ($p = 0.101$), so the plates did not differ by a gross shift in signal.

Both directions of the threshold sensitivity analysis were examined, and are displayed in Figure 6. Applying the lower control value of 0.203 to all 33 isolates gives 26 positives (78.8%, 95% CI 62.2–89.3); applying the higher value of 0.225 to all 33 gives 25 positives (75.8%, 95% CI 59.0–87.2), identical to the primary estimate. The primary outcome is therefore stable across the full range of control values actually used, varying by a single isolate.

The ordinal outcome proved more stable still, as Figure 6B shows. Under a single control value of 0.203 the moderate-and-strong proportion is 23/33 (69.7%, 95% CI 52.7–82.6); under a single value of 0.225 it is 23/33 (69.7%, 95% CI 52.7–82.6) — the same figure as the primary analysis in both cases. The distribution of categories did not differ significantly between the two assay plates (Mann–Whitney $p = 0.119$; rank-biserial $r = 0.305$), nor did the moderate-and-strong proportion (82.4% on plate 1 against 56.3% on plate 2; odds ratio 3.49, 95% CI 0.60–26.53; $p = 0.141$). The plate effect is thus confined to the binary threshold and does not propagate to the ordinal outcome on which the study's principal claim rests.

Two further checks bear on the plate effect. The species composition of the two plates differed — all four *P. mirabilis* isolates fell on plate 2 ($p = 0.044$), as did both non-adherent *E. coli* isolates — so part of the difference could in principle be compositional. Restricting the comparison to *P. aeruginosa* alone, however, the difference persists: 10 of 11 isolates on plate 1 were positive (90.9%) against two of six on plate 2 (33.3%; p

$= 0.028$). The effect is therefore not explained by species composition. A model adjusting simultaneously for plate and organism was not fitted, because 33 isolates across seven species cannot support it. Only 1 isolate lay within 10% of its threshold and 2 within 20%, so the borderline population is small.

Presented without commitment to a single cut-off, as shown in Figure 6A, the proportion of isolates exceeding a biofilm index of 0.75, 1.0, 1.5, 2.0, 3.0 and 4.0 was 81.8%, 75.8%, 75.8%, 69.7%, 51.5% and 30.3% respectively. The estimate is flat between indices of 1.0 and 1.5, which is the region in which the choice of control value operates, and this is the quantitative basis for regarding the primary estimate as threshold-robust. Finally, comparing the biofilm index between the two functional groups as a continuous variable, Enterobacterales had a higher median index than non-fermenting Gram-negative bacilli (4.55 against 2.78; Hodges–Lehmann difference 1.62; Mann–Whitney $p = 0.095$; rank-biserial $r = 0.373$). The difference is in the direction that the species-level results in Table 2 suggest but does not reach significance, and as one of the exploratory comparisons it does not survive adjustment. Every value reported above can be checked independently, the complete participant-level dataset — age, sex, cleaning frequency, indication, organism, biofilm status, mean OD₅₇₀ and the applicable control value for each of the 33 isolates — is reproduced in Table 5. Applying the classification rule of the Methods to those optical densities reproduces the biofilm status reported here for 33 of 33 isolates.

Table 1. Demographic and clinical characteristics of 33 patients with a tracheostomy tube in situ for four weeks, Dr. M. Djamil General Hospital, Universitas Andalas, Padang, West Sumatra, January–July 2024.

Characteristic	n	%	95% CI
Sex			
Male	21	63.6	46.6–77.8
Female	12	36.4	22.2–53.4
Age, years			
Mean $\pm$ SD	55.64 $\pm$ 12.79	—	51.1–60.2 ^a
Median (IQR)	56 (47–66)	—	—
Range	26–88	—	—
Age group, years			
20–29	1	3.0	0.5–15.3
30–39	1	3.0	0.5–15.3
40–49	9	27.3	15.1–44.2
50–59	12	36.4	22.2–53.4
≥ 60	10	30.3	17.4–47.3
Indication for tracheostomy			
Upper-airway obstruction	19	57.6	40.8–72.8
Prolonged endotracheal intubation	14	42.4	27.2–59.2
Inner-cannula cleaning frequency			
Twice daily	12	36.4	22.2–53.4
Three or more times daily	21	63.6	46.6–77.8
Device age at sampling			
4 weeks (protocol-fixed)	33	100.0	89.6–100 ^b
Specimens yielding bacterial growth	33	100.0	89.6–100 ^b

^a 95% confidence interval for the mean. ^b Clopper–Pearson interval, used at the 100% boundary where the Wilson interval is not defined upward.

Percentages are of the 33 enrolled patients. Comorbidity, prior antibiotic exposure, ethnicity, residence, socioeconomic status and educational attainment were not recorded in the case-record form and are therefore not reported.

Table 2. Bacterial species isolated from tracheostomy cannula swabs, with in-vitro biofilm production by species (n = 33 isolates).

Organism	n	%	95% CI	Gram-stain class	Biofilm-positive	Biofilm index, median (IQR)
<i>Pseudomonas aeruginosa</i>	17	51.5	35.2–67.5	Gram-negative bacillus	12/17 (70.6%)	3.31 (1.00–3.91)
<i>Klebsiella pneumoniae</i>	4	12.1	4.8–27.3	Gram-negative bacillus	4/4 (100.0%)	5.08 (4.56–5.98)
<i>Proteus mirabilis</i>	4	12.1	4.8–27.3	Gram-negative bacillus	4/4 (100.0%)	4.24 (3.56–5.02)
<i>Acinetobacter baumannii</i>	3	9.1	3.1–23.6	Gram-negative bacillus	2/3 (66.7%)	2.21 (1.19–2.49)
<i>Escherichia coli</i>	2	6.1	1.7–19.6	Gram-negative bacillus	0/2 (0.0%)	0.36 (0.12–0.61)
<i>Staphylococcus aureus</i>	2	6.1	1.7–19.6	Gram-positive coccus	2/2 (100.0%)	2.09 (1.97–2.20)
<i>Enterobacter cloacae</i>	1	3.0	0.5–15.3	Gram-negative bacillus	1/1 (100.0%)	5.65 (5.65–5.65)
Gram-negative bacilli, total	31	93.9	80.4–98.3	—	23/31 (74.2%)	3.31 (1.35–4.65)
Gram-positive cocci, total	2	6.1	1.7–19.6	—	2/2 (100.0%)	2.09 (1.97–2.20)
Non-fermenting Gram-negative bacilli	20	60.6	43.7–75.3	—	14/20 (70.0%)	2.78 (0.93–3.80)
Enterobacterales	11	33.3	19.8–50.4	—	9/11 (81.8%)	4.55 (2.74–5.37)

One predominant isolate was recorded per patient, so species percentages sum to 100% and polymicrobial colonisation could not be assessed. Confidence intervals are Wilson score intervals. The biofilm index is the mean optical density at 570 nm divided by the plate-specific optical-density control. Non-fermenting Gram-negative bacilli comprise *P. aeruginosa* and *A. baumannii*; Enterobacterales comprise *K. pneumoniae*, *P. mirabilis*, *E. coli* and *E. cloacae*. Species identification was by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (Biomérieux VITEK MS). Antimicrobial susceptibility testing was not performed in this study; no susceptibility or resistance data are reported anywhere in this manuscript. Culture, identification and biofilm phenotyping: Department of Clinical Microbiology, Faculty of Medicine, Universitas Andalas.

Table 3. Quantitative biofilm phenotype of 33 tracheostomy cannula isolates by the tissue-culture-plate assay, including the assay-plate sensitivity analysis.

Biofilm measure	n	%	95% CI	Biofilm index
Biofilm production (binary)				
Positive (OD > ODc)	25	75.8	59.0–87.2	—
Negative (OD ≤ ODc)	8	24.2	12.8–41.0	—
Biofilm strength (Stepanović four-tier)				
Non-adherent (OD ≤ ODc)	8	24.2	12.8–41.0	0.58
Weak (ODc < OD ≤ 2 × ODc)	2	6.1	1.7–19.6	1.79
Moderate (2 × ODc < OD ≤ 4 × ODc)	13	39.4	24.7–56.3	3.01
Strong (OD > 4 × ODc)	10	30.3	17.4–47.3	5.15
Moderate or strong, combined	23	69.7	52.7–82.6	3.91
Biofilm index (OD570 / ODc)				
Median (IQR)	—	—	—	3.01 (1.71–4.55)
Range	—	—	—	–0.12 to 8.67
By assay plate				
Plate 1, isolates 1–17 (ODc 0.203)	16/17	94.1	73.0–99.0	3.58
Plate 2, isolates 18–33 (ODc 0.225)	9/16	56.3	33.2–76.9	2.34
Sensitivity analysis: single ODc of 0.203 applied to all 33	26/33	78.8	62.2–89.3	—

ODc, optical-density control, calculated as the mean optical density of the negative control plus three standard deviations. Categories follow Stepanović and colleagues, as specified in the study protocol. The biofilm index is the isolate's mean optical density divided by the optical-density control of its own plate; a value of 1.0 corresponds exactly to the detection threshold. Two control values were used across the run, and the plate comparison is presented so that readers can judge its influence; the sensitivity row re-classifies all 33 isolates against the single lower control value. Median values are shown for each stratum.

Table 4. Association of patient, care and microbiological factors with in-vitro biofilm production (n = 33).

Factor	Biofilm-positive, n/N (%)	95% CI	Odds ratio (95% CI)	p	Cramér's V	Holm-adjusted p
Pre-specified comparisons						
Sex						
Male	17/21 (81.0%)	60.0–92.3	2.07 (0.30–14.48)	0.420	0.160	0.619
Female (reference)	8/12 (66.7%)	39.1–86.2	1.00 (reference)	—	—	—
Inner-cannula cleaning frequency						
Twice daily	11/12 (91.7%)	64.6–98.5	5.26 (0.54–269.98)	0.206	0.281	0.619
Three or more times daily (reference)	14/21 (66.7%)	45.4–82.8	1.00 (reference)	—	—	—
Indication for tracheostomy						
Upper-airway obstruction	16/19 (84.2%)	62.4–94.5	2.86 (0.44–22.97)	0.238	0.230	0.619
Prolonged intubation (reference)	9/14 (64.3%)	38.8–83.7	1.00 (reference)	—	—	—
Exploratory comparisons						
Age						
≥60 years	8/10 (80.0%)	49.0–94.3	1.40 (0.19–17.17)	1.000	0.065	1.000
<60 years (reference)	17/23 (73.9%)	53.5–87.5	1.00 (reference)	—	—	—
Organism group						
Non-fermenting Gram-negative bacilli	14/20 (70.0%)	48.1–85.5	0.43 (0.04–3.09)	0.431	0.167	1.000
Enterobacterales or S. aureus (reference)	11/13 (84.6%)	57.8–95.7	1.00 (reference)	—	—	—
Dominant species						
P. aeruginosa	12/17 (70.6%)	46.9–86.7	0.56 (0.07–3.66)	0.688	0.124	1.000
All other organisms (reference)	13/16 (81.3%)	57.0–93.4	1.00 (reference)	—	—	—
Exploratory multivariable model (Firth penalised logistic regression; 3 covariates, 8 minority events, EPV 2.7)						
Male sex	—	—	0.69 (0.08–6.04)	0.734	—	—
Cleaning 2x/day (vs ≥3x/day)	—	—	3.63 (0.44–29.87)	0.231	—	—
UAO indication (vs prolonged intubation)	—	—	2.54 (0.33–19.78)	0.373	—	—
Model fit: Nagelkerke R ² = 0.170; likelihood-ratio p = 0.262; Hosmer–Lemeshow χ ² = 2.41, df = 2, p = 0.300						

Odds ratios are conditional maximum-likelihood estimates with exact 95% confidence intervals; p-values are from two-sided Fisher's exact tests. Cramér's V is reported as the effect size. Holm adjustment was applied separately within the three pre-specified and the six exploratory comparisons. The multivariable model uses Firth penalisation because eight minority events across three covariates give an events-per-variable ratio of 2.7, far below the conventional minimum of ten; its estimates are exploratory and are not the basis of any conclusion. Odds ratios above one indicate higher odds of biofilm production in the first-listed category. Statistical analysis: Department of Public Health and Community Medicine, Faculty of Medicine, Universitas Andalas.

Table 5. Complete participant-level dataset for all 33 isolates.

ID	Age (y)	Sex	Inner-cannula cleaning	Indication	Organism	Mean OD570	ODc	Biofilm index	Biofilm status	Category
1	49	F	≥3×/day	UAO	P. aeruginosa	0.764	0.203	3.76	Positive	Moderate
2	49	F	≥3×/day	PEI	A. baumannii	0.562	0.203	2.77	Positive	Moderate
3	56	F	≥3×/day	PEI	P. aeruginosa	0.120	0.203	0.59	Negative	Non-adherent
4	72	F	≥3×/day	PEI	P. aeruginosa	1.061	0.203	5.23	Positive	Strong
5	72	M	2×/day	UAO	S. aureus	0.469	0.203	2.31	Positive	Moderate
6	43	M	≥3×/day	UAO	S. aureus	0.378	0.203	1.86	Positive	Weak
7	26	F	≥3×/day	PEI	P. aeruginosa	0.794	0.203	3.91	Positive	Moderate
8	68	M	2×/day	UAO	P. aeruginosa	0.671	0.203	3.31	Positive	Moderate
9	44	F	≥3×/day	PEI	K. pneumoniae	1.760	0.203	8.67	Positive	Strong
10	41	F	≥3×/day	PEI	K. pneumoniae	1.030	0.203	5.07	Positive	Strong
11	66	M	2×/day	UAO	P. aeruginosa	0.830	0.203	4.09	Positive	Strong
12	56	M	2×/day	UAO	K. pneumoniae	1.031	0.203	5.08	Positive	Strong
13	57	M	2×/day	UAO	P. aeruginosa	0.727	0.203	3.58	Positive	Moderate
14	44	M	2×/day	UAO	P. aeruginosa	0.347	0.203	1.71	Positive	Weak
15	88	M	2×/day	PEI	P. aeruginosa	1.380	0.203	6.80	Positive	Strong
16	56	M	2×/day	UAO	P. aeruginosa	0.526	0.203	2.59	Positive	Moderate
17	47	M	2×/day	UAO	P. aeruginosa	0.566	0.203	2.79	Positive	Moderate
18	53	M	≥3×/day	PEI	E. coli	0.191	0.225	0.85	Negative	Non-adherent
19	43	F	2×/day	PEI	P. aeruginosa	0.168	0.225	0.75	Negative	Non-adherent
20	36	F	≥3×/day	PEI	P. aeruginosa	0.745	0.225	3.31	Positive	Moderate
21	67	M	≥3×/day	UAO	P. mirabilis	1.447	0.225	6.43	Positive	Strong
22	42	M	2×/day	UAO	P. mirabilis	0.882	0.225	3.92	Positive	Moderate
23	59	M	≥3×/day	UAO	P. mirabilis	0.554	0.225	2.46	Positive	Moderate
24	54	M	≥3×/day	UAO	P. mirabilis	1.024	0.225	4.55	Positive	Strong
25	71	M	≥3×/day	UAO	A. baumannii	0.037	0.225	0.16	Negative	Non-adherent
26	73	F	≥3×/day	UAO	P. aeruginosa	0.126	0.225	0.56	Negative	Non-adherent
27	70	F	≥3×/day	PEI	K. pneumoniae	0.678	0.225	3.01	Positive	Moderate
28	51	M	2×/day	PEI	A. baumannii	0.497	0.225	2.21	Positive	Moderate
29	52	M	≥3×/day	UAO	P. aeruginosa	0.040	0.225	0.18	Negative	Non-adherent
30	55	M	≥3×/day	PEI	P. aeruginosa	0.224	0.225	1.00	Negative	Non-adherent
31	58	F	≥3×/day	PEI	E. coli	−0.028	0.225	−0.12	Negative	Non-adherent
32	56	M	≥3×/day	UAO	P. aeruginosa	1.068	0.225	4.75	Positive	Strong
33	62	M	≥3×/day	UAO	E. cloacae	1.272	0.225	5.65	Positive	Strong

UAO, tracheostomy for upper-airway obstruction; PEI, tracheostomy for prolonged endotracheal intubation; ODc, plate-specific optical-density control. The biofilm index is the mean optical density divided by the ODc of the isolate's own plate. Category follows the four-tier classification defined in the Methods. Isolates 1–17 were read against ODc = 0.203 and isolates 18–33 against ODc = 0.225. Applying the classification rule to the optical densities in this table reproduces the biofilm status shown for 33 of 33 isolates.

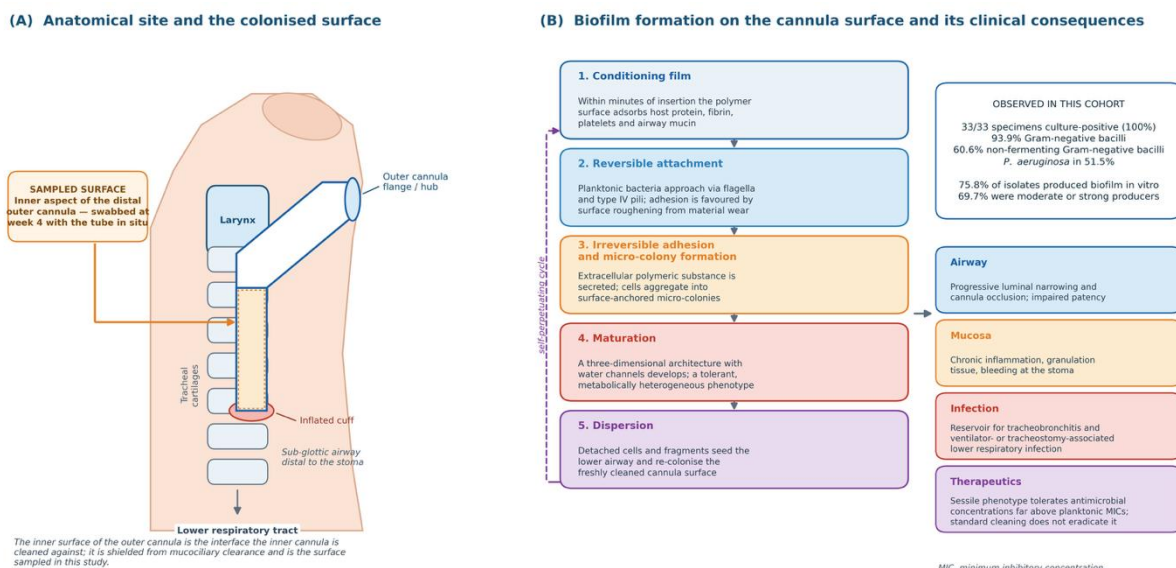


Figure 1. (A) Anatomical site of sampling — the inner aspect of the distal outer cannula, the surface shielded from mucociliary clearance — and (B) the biofilm life cycle on that surface with its clinical consequences, annotated with the findings of the present cohort.

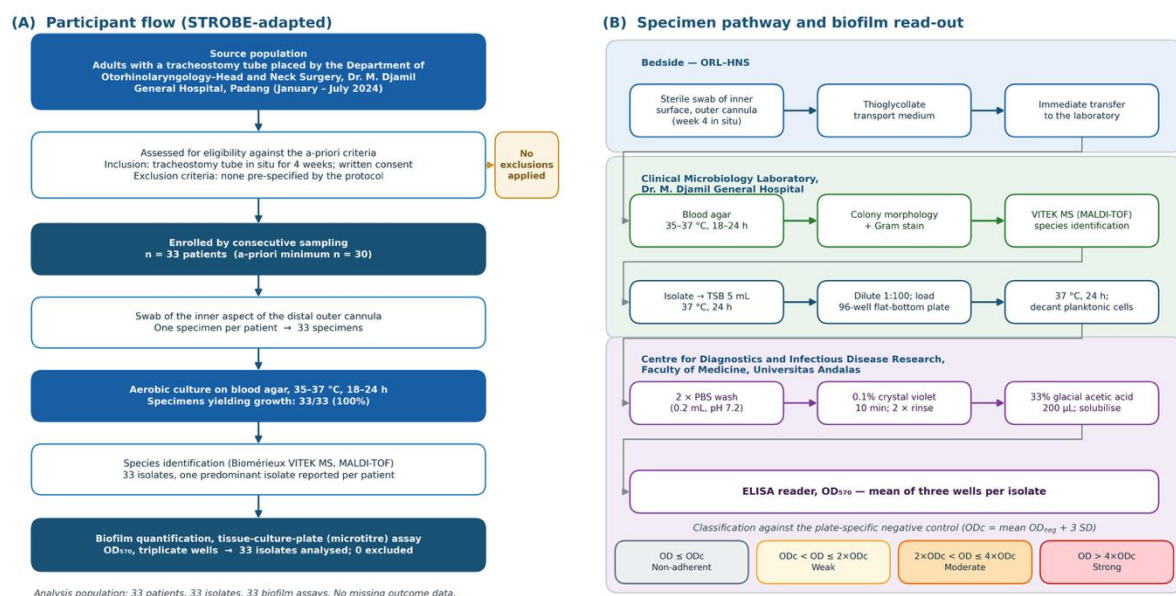


Figure 2. (A) Participant selection flow, adapted from the STROBE cross-sectional checklist, and (B) the specimen pathway from bedside sampling to the optical-density read-out, showing the three units involved and the classification thresholds applied.

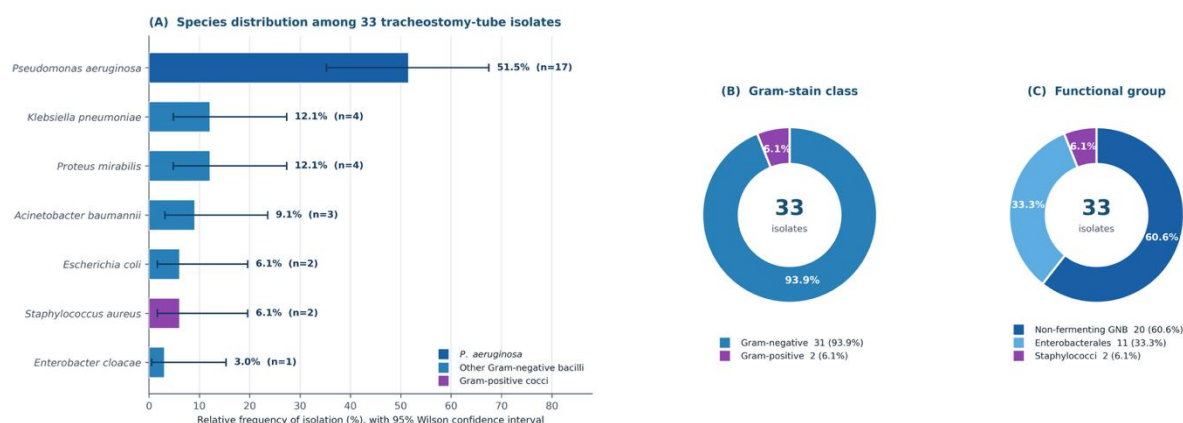


Figure 3. (A) Distribution of the seven bacterial species isolated from tracheostomy cannula swabs, with 95% Wilson confidence intervals; (B) Gram-stain class; and (C) functional group, at Dr. M. Djamil General Hospital, Universitas Andalas, Padang, West Sumatra (n = 33 isolates).

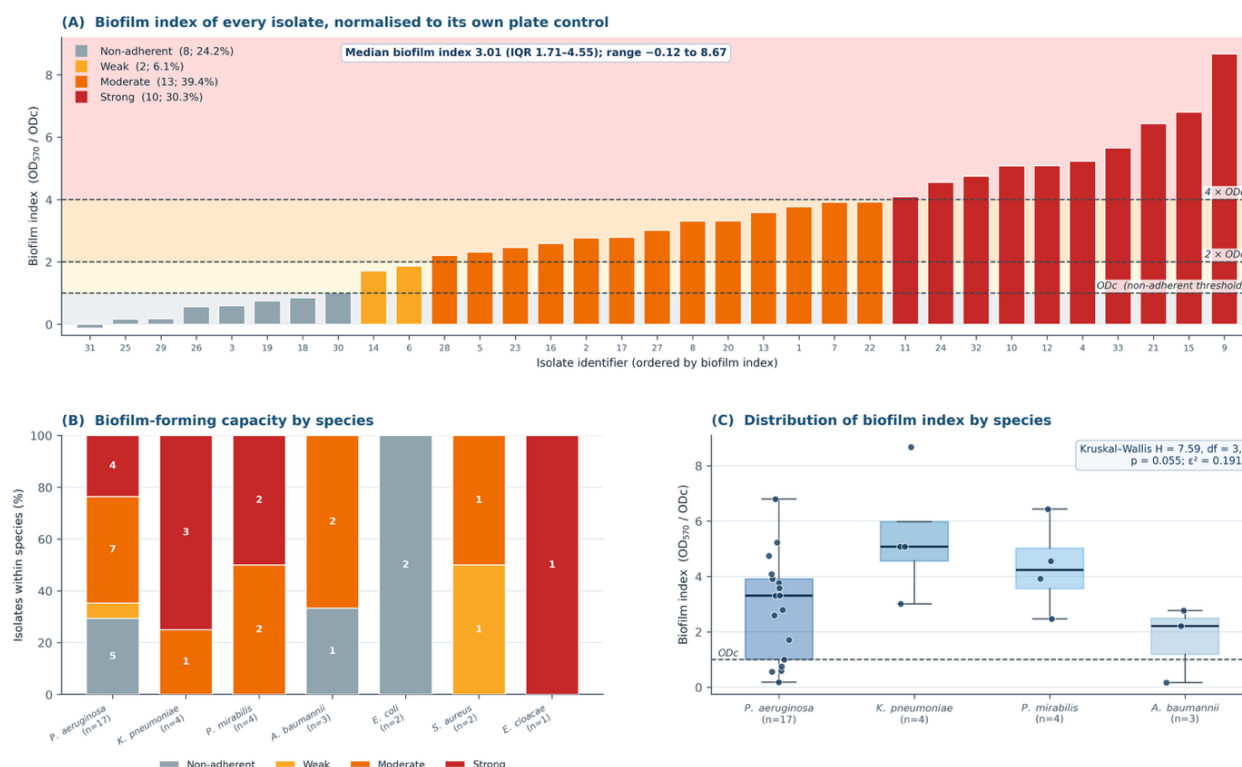


Figure 4. Quantitative biofilm phenotype. (A) Biofilm index of every isolate, normalised to its own plate control and ordered by magnitude, with the classification thresholds shown; (B) distribution of biofilm-strength category within each species; and (C) distribution of the biofilm index across the four species contributing three or more isolates.

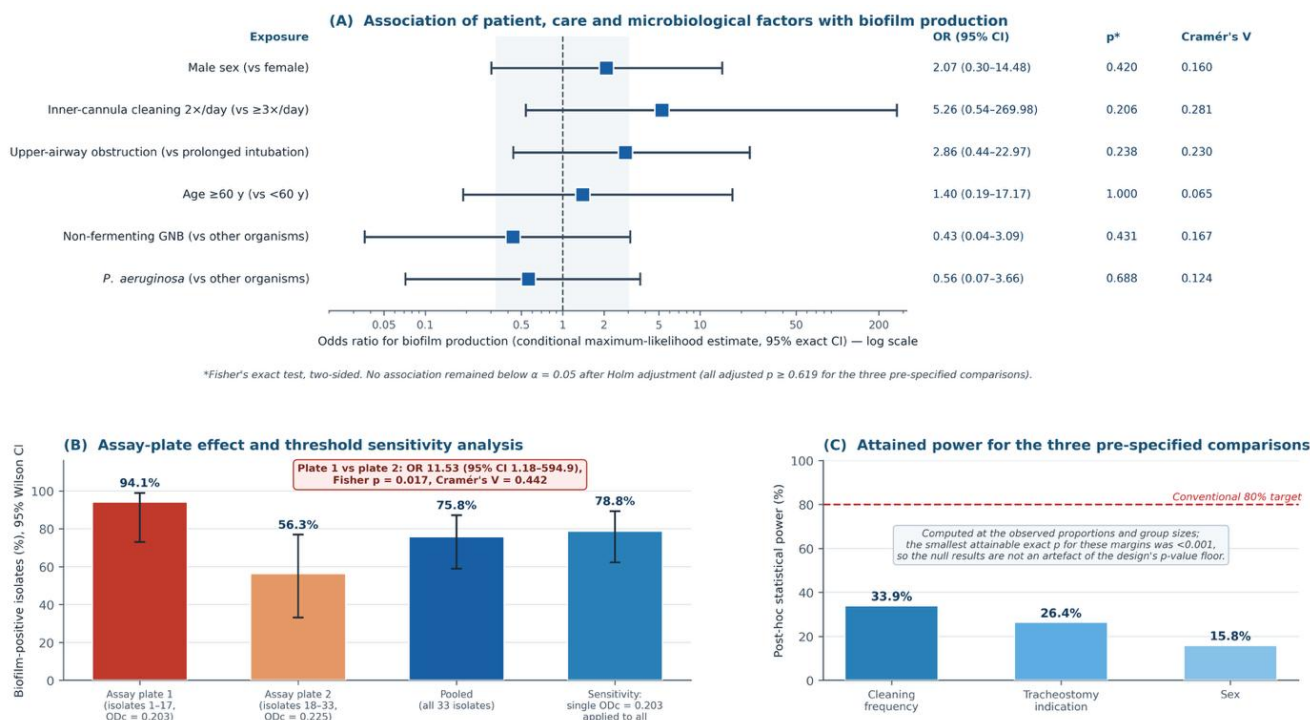


Figure 5. (A) Forest plot of the association between patient, care and microbiological factors and in-vitro biofilm production, with conditional odds ratios, exact 95% confidence intervals, exact p-values and Cramér's V; (B) biofilm positivity by assay plate, with the sensitivity analysis applying a single optical-density control to all isolates; and (C) attained post-hoc power for the three pre-specified comparisons.

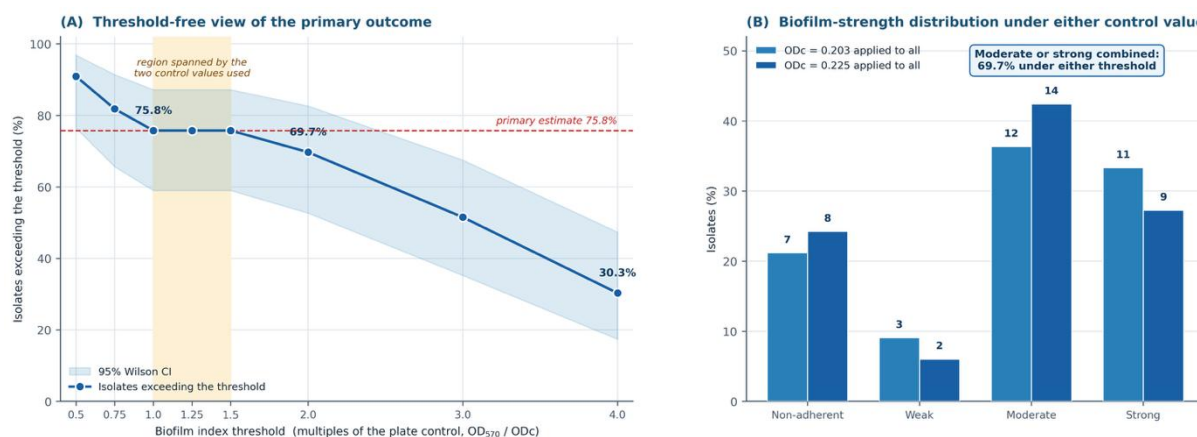


Figure 6. Threshold robustness of the primary and secondary outcomes. (A) Proportion of isolates exceeding a range of candidate biofilm-index thresholds, with 95% Wilson confidence intervals; the shaded band marks the region spanned by the two optical-density control values actually used. (B) Distribution of biofilm-strength category when each control value in turn is applied to all 33 isolates.

4. Discussion

Principal findings

In 33 adults whose tracheostomy tube had been in place for exactly four weeks at the tertiary otorhinolaryngology referral centre for West Sumatra, every cannula swab yielded bacterial growth, almost all of it Gram-negative. Non-fermenting Gram-negative bacilli accounted for three in five isolates and *P. aeruginosa* alone for half, as summarised in Table 2 and Figure 3. Three-quarters of the isolates formed biofilm in vitro and — the finding that the binary result conceals — 69.7% did so at moderate or strong intensity, as quantified in Table 3 and Figure 4. No patient characteristic, indication for tracheostomy or reported cleaning frequency predicted which isolates would do so (Table 4). These are the first data of their kind from Sumatra, and to our knowledge the first from anywhere to fix the device age at sampling so that the estimate refers to a defined point in the life of the tube.

The bacteriological profile in context

The species distribution replicates the international pattern closely. The finding that *P. aeruginosa* accounted for 51.5% of isolates (Table 2) sits directly alongside the meta-analytic result that *Pseudomonas* is significantly more prevalent on tracheostomy tubes than on endotracheal tubes, and above the proportions reported in individual prospective series; the confidence interval around the present estimate (35.2–67.5%) overlaps those series, so the difference is compatible with sampling variation in a cohort of this size rather than evidence of a distinct local flora. The pooled finding that *Pseudomonas* is device-specific in this way carries an operational corollary: endotracheal-tube surveillance is not a substitute for tracheostomy-tube surveillance.^{1,5,6}

The predominance of Gram-negative bacilli at 93.9% and of non-fermenters at 60.6% is the clinically consequential summary. A tracheostomy cannula is, in effect, a selective environment: it bypasses the nasal

filtration and mucociliary clearance that keep the lower airway sterile, and it presents a moist polymer surface on which organisms tolerant of desiccation, of low nutrient availability and of disinfectants are advantaged.^{17,35,36} *P. aeruginosa*, which combines minimal nutritional requirements with quorum-sensing control of exopolysaccharide production and a broad efflux repertoire, is precisely the organism such an environment selects.^{11,12} The tropical humidity of West Sumatra plausibly reinforces this, but the present study has no comparison climate and cannot test it. What can be said without qualification is that the organisms recovered are those for which resistant phenotypes carry the greatest global burden, and that they were recovered from a device that opens directly onto the lower respiratory tract.^{8,13}

How much biofilm, and how strong

The prevalence of 75.8% sits between the two poles of the published literature and differs significantly from each, as reported in the benchmarking analysis above. It is higher than the 57% reported by Raveendra and colleagues, who used the same microtitre-plate assay on the same specimen type ($p = 0.034$), and lower than the figures above 90% reported from Polish inpatient and outpatient cohorts ($p = 0.014$).^{2,20} The direction of both differences is explicable. Studies that examine the tube surface directly detect biofilm that is present on the device, whereas the microtitre assay measures whether a recovered isolate can form biofilm on polystyrene under standard laboratory conditions; the former will systematically exceed the latter, because an organism may participate in a mixed-species biofilm in vivo without being an efficient monospecies former in vitro (Figure 1B).^{1,22} Conversely, the higher figure here than in the Indian series may reflect the fixed four-week device age, since biofilm accumulates with time in situ and series with mixed or shorter device ages will dilute mature cases with immature ones.²⁵ This is the argument for fixing device age by design, and it is the

methodological contribution of the present study as much as the epidemiological one.

The more substantive result is the one that the binary outcome discards. Applying the four-tier classification that the assay supports and that the protocol already specified, 69.7% of isolates (95% CI 52.7–82.6) were moderate or strong producers and 30.3% were strong (Table 3, Figure 4A). A cohort in which every positive isolate is weak and a cohort in which every positive isolate is strong report an identical binary prevalence and present entirely different clinical problems, because matrix density governs both antimicrobial penetration and the mechanical difficulty of removal.^{10,24} Reporting only the binary outcome, as most of the tracheostomy literature does, therefore discards the information most relevant to the decision the clinician actually faces. We would encourage future device-biofilm studies to report the ordinal classification as standard; it requires no additional laboratory work, since the optical densities are already collected.

Species differences in biofilm capacity

All four *K. pneumoniae* and all four *P. mirabilis* isolates produced biofilm, and the highest median indices belonged to those two species, whereas *P. aeruginosa* — the numerically dominant organism — was positive in 70.6% and neither *E. coli* isolate was positive at all, as Table 2 and Figure 4B record. The species difference across the four best-represented organisms approached but did not reach conventional significance ($p = 0.055$, $\epsilon^2 = 0.191$). Three cautions are necessary before this is interpreted. The denominators are three and four isolates for most species, so a 100% proportion carries a Clopper–Pearson lower bound of 39.8% and is compatible with a true value below one-half. The Kruskal–Wallis result is one of six exploratory tests and does not survive Holm adjustment (adjusted $p = 0.331$). And a p -value of 0.055 is not a significant result reported as nearly significant; it is a non-significant result in a small sample.

With those cautions, the direction is biologically coherent. *K. pneumoniae* is a notably efficient former on abiotic surfaces, and clinical isolates from tracheostomy tubes combine biofilm capacity with virulence and resistance determinants, a pattern also reported for multidrug-resistant *K. pneumoniae* from Indonesian tertiary practice.^{2,14,37,38} That the dominant organism in the cohort was not the most reliable biofilm former is a useful corrective to the assumption that the two properties travel together: *P. aeruginosa* dominates the cannula because it colonises the airway of these patients readily, not because it is uniquely adherent, and an empirical strategy directed only at *Pseudomonas* would leave the strongest biofilm formers in the cohort unaddressed. If that asymmetry is real, a further consequence follows as a hypothesis for testing: species identity alone is a poor predictor of biofilm burden, so surveillance that records only the

organism will not capture the property that matters clinically.

The single striking negative finding is that neither *E. coli* isolate produced biofilm. With two isolates this establishes nothing; the upper confidence bound is 84.2%. It is recorded because selective omission of null species results is one of the ways small microbiological series mislead.

Why no factor predicted biofilm, and what that means

The three pre-specified associations in Table 4 were all null after adjustment, and it matters to distinguish between the two very different statements this could support. The first — that these factors do not influence biofilm formation — is not supported. Attained power was 33.9%, 26.4% and 15.8% for the cleaning, indication and sex comparisons (Figure 5C); a study with 16% power to detect the effect it observed cannot exclude that effect. The confidence intervals make the same point more directly: the interval for cleaning frequency runs from 0.54 to 270.0, and is compatible both with a halving of the odds and with a very large increase. The second statement — that this study did not detect an association — is what the data support, and it is all that should be drawn from them. We note that the study was not incapable of returning a significant result: enumeration over the observed marginal totals shows that the smallest attainable exact p was below 0.001 for each comparison.

The cleaning-frequency result nonetheless deserves comment because its direction is counter-intuitive. Biofilm was more frequent where the inner cannula was cleaned twice daily (91.7%) than where it was cleaned three or more times daily (66.7%), an unadjusted odds ratio of 5.26. Taken at face value this would suggest that more frequent cleaning reduces biofilm, which is biologically plausible and consistent with randomised and prospective evidence that chlorhexidine decontamination reduces biofilm-forming colony growth on the cannula.^{3,4} But the variable is self-reported by patients and carers, is subject to social-desirability bias, and records frequency without recording technique, agent or duration. It is also open to reverse causation, and the mechanism is clinically familiar: carers clean more often when the tube is visibly soiled or the patient is producing more secretions, so reported cleaning frequency is partly a marker of secretion burden rather than an independent exposure, and the association it produces may run in either direction. The honest conclusion is that cleaning frequency as recorded here is too crude an instrument to answer the question, and that a study designed to answer it would need observed rather than reported technique, a defined cleaning agent, and biofilm quantified before and after. The care recommendation made below does not rest on this association, which we have just set aside, but on the randomised and prospective decontamination evidence.^{4,35}

The assay-plate effect

The largest single association in this dataset is not clinical. Isolates read against the first plate's control value were biofilm-positive in 94.1% of cases against 56.3% for the second plate (odds ratio 11.53, 95% CI 1.18–594.9; $p = 0.017$; Cramér's $V = 0.442$), as Figure 5B shows. Reporting this is uncomfortable and necessary, because a reader who is not told will infer that all 33 isolates were classified against a single threshold. It is also not an anomaly peculiar to this study: interlaboratory work on microtitre-plate biofilm quantification has shown that a substantial share of the variance in such assays is attributable to run and laboratory rather than to the organism.²³ One further consideration bears on it. Because OD_c is the mean negative-control optical density plus three standard deviations, the threshold is a function of that standard deviation, and with a small number of control wells the three-standard-deviation rule is itself unstable; this is a plausible partial explanation for the difference between the two plates. A second consequence, of the missing positive control, should also be stated here rather than only in the Limitations: without a reference biofilm-forming strain on each plate there is no internal check distinguishing a plate whose isolates genuinely adhered less from a plate on which the staining or solubilisation step underperformed.

Three things bound its importance further. First, and decisively for the paper's principal claim, the plate effect does not carry to the ordinal outcome: the distribution of categories did not differ significantly between plates ($p = 0.119$), and the moderate-and-strong proportion of 69.7% is reproduced exactly when either control value is applied to all 33 isolates (Figure 6B). The figure that appears in the abstract and the conclusion is therefore threshold-invariant across the range of values actually used, which is a stronger statement than robustness of the binary estimate alone. Second, the effect is not an artefact of species composition. The two plates did differ in composition — all four *P. mirabilis* isolates and both non-adherent *E. coli* isolates fell on the second — but the difference persists within *P. aeruginosa* alone (90.9% against 33.3%; $p = 0.028$). Whatever produced it operated on isolates of the same species. Third, only one isolate lies within 10% of its threshold, so this is not a case of many ambiguous readings being reallocated; it is a modest shift in the position of the distribution relative to a fixed cut-off, which is exactly what a dichotomy is least able to absorb and a continuous index is designed to tolerate.²⁴

The practical implication for future work is that a single negative-control set should be run across an entire study wherever possible, that the control value should always be reported, and that the continuous index rather than the dichotomy should carry the analysis — all three of which the present analysis adopts. The general point is not specific to this study: where a graded classification and a dichotomy are derived from

the same continuous measurement, it is the dichotomy that is fragile to a shift in the cut-off, and the graded classification that is not.

Clinical and public-health implications

Three implications follow, and each should be read at the level of confidence the data support. The first concerns tube-exchange scheduling. That a predominantly non-fermenting Gram-negative flora with a moderate-to-strong biofilm phenotype in 69.7% of isolates is established by week four supports scheduled exchange of the tracheostomy tube at approximately that interval, which coincides with the lower bound of the exchange window discussed in the clinical literature.²⁵ Two independent lines of reasoning converging on the same interval is a stronger basis for practice than either alone. Its limits should be stated in the same breath. These data show that a dense Gram-negative biofilm-forming flora is present at four weeks; they do not show that it was absent at three weeks, because no earlier point was sampled, and they do not show that exchanging at four weeks improves any clinical outcome, because no outcome was measured and no interval was randomised. The recommendation is also falsifiable in a specific way: if a longitudinal study found a comparable biofilm burden at week two, the case for four weeks would weaken considerably. Finally, tube exchange is not itself risk-free, particularly through an immature or difficult stoma; a four-week schedule should be understood as a default to be varied on clinical grounds rather than as a mandate.

The second concerns cannula care. Once a mature biofilm is established, routine mechanical cleaning does not eradicate it.¹⁰ The practical consequences are that carer education should be framed around limiting accumulation rather than achieving sterility, and that the choice of decontamination agent matters: chlorhexidine has both randomised and prospective observational support on this specific device, whereas disinfectants differ markedly in their capacity to eradicate established Gram-negative biofilm.^{3,4,35} This has particular force in the present setting, where much tracheostomy care after discharge is delivered at home by family members. Carer education at this centre covers removal and cleaning of the inner cannula with a purpose-designed brush and soapy water followed by a warm rinse and air drying, humidification, suctioning technique, stoma hygiene, and recognition of the signs that require urgent review; what the present findings add is the message that this regimen limits accumulation and cannot be expected to sterilise the device, so adherence should be judged by consistency rather than by the appearance of the cannula.

The third concerns antimicrobial stewardship, and here the study's limits must be stated as clearly as its findings. Indonesia's national response to antimicrobial resistance rests on surveillance and stewardship, and the national surveillance network depends on locally generated data of exactly the kind

reported here.^{27,28} These data establish which organisms occupy the cannula niche in West Sumatra and how readily they adopt a biofilm phenotype. A distinction should be drawn between the two for policy purposes: the species distribution is the kind of information surveillance systems already collect and can act on, whereas the biofilm phenotype is collected by no surveillance system at present and has no agreed reporting standard, so its policy relevance is prospective rather than immediate. Neither establishes which antibiotic to use, because no susceptibility testing was performed. It would be a serious misreading of this paper to derive an empirical regimen from it. What the paper does support is the case for the next study: the published association between the biofilm phenotype and multidrug resistance means that a cohort of this composition is precisely where paired biofilm phenotyping and susceptibility testing to CLSI or EUCAST breakpoints, with multidrug resistance defined by the Magiorakos criteria, would be most informative, and a cumulative antibiogram of the kind now published from other Indonesian tertiary centres is the natural output, including for the older patients who make up much of this cohort.^{34,39-41}

Strengths

The study has four strengths. Device age was fixed by protocol at four weeks rather than observed, so the prevalence estimate refers to a defined point in the life of the tube and is directly usable for exchange scheduling; to our knowledge no previous tracheostomy-cannula series has done this. Species identification was by MALDI-TOF mass spectrometry rather than by biochemical panel, which removes a common source of misidentification among non-fermenting Gram-negative bacilli. The biofilm assay was the reference phenotypic method, performed in triplicate, with a plate-specific negative control that was recorded and is reported, allowing every classification to be reconstructed from the raw optical densities in Table 5, which publishes the complete dataset with the article and allows any reader to reclassify the isolates under assumptions of their own.²¹

Limitations

The limitations are substantial and are set out in full. The most important is that no antimicrobial susceptibility testing was performed, so the study cannot link the biofilm phenotype to resistance, cannot report multidrug resistance, methicillin resistance or extended-spectrum β -lactamase production, and cannot inform empirical prescribing; this is the single largest gap and the priority for the next study.^{34,39} Second, one predominant isolate was recorded per patient, so polymicrobial colonisation could not be assessed, and since tracheostomy-tube biofilms are frequently polymicrobial the community complexity is almost certainly understated.¹ Third, the microtitre assay measures the capacity of a recovered isolate to

form biofilm on polystyrene *in vitro*; it does not visualise biofilm on the cannula, so the study describes biofilm-forming capacity rather than biofilm presence on the device.²² Fourth, the case-record form did not capture comorbidity, immune status, prior or current antibiotic exposure, tube brand, size or material, or duration of hospitalisation. Fifth, the sample of 33 is adequate for the primary prevalence estimate, whose confidence interval has a half-width of 14.1 percentage points, but is small for subgroup comparison; the multivariable model has an events-per-variable ratio of 2.7 and is exploratory only. Sixth, cleaning frequency was self-reported and unverified. Seventh, the study was conducted at a single tertiary referral centre over seven months, so the flora may not generalise to primary or secondary centres, to other Indonesian provinces or to other seasons. Eighth, two optical-density control values were used across the run; the sensitivity analyses show the primary estimate to be robust, but residual misclassification near the threshold cannot be eliminated.²³ Ninth, the quality-assurance procedure records storage of specimens at -80°C but does not state at which stage relative to culture and to the assay, so the number of freeze-thaw cycles preceding biofilm quantification is unknown; freezing is known to affect subsequent biofilm assay performance, and this is an uncontrolled source of variation.

Several further limitations were identified during peer review. The first, and the most important after the absence of susceptibility testing, is a survivorship effect inherent to the design: because only patients still under departmental follow-up at week four could be sampled, those who died, were decannulated early or were lost to follow-up are absent by construction. If early decannulation is associated with a lower secretion burden and hence less biofilm, the estimate reported here is biased upward. Beyond that, the tracheostomy tubes themselves are not characterised: brand, material, size, cuff status and whether any tube had been reprocessed were not recorded, and since surface properties govern bacterial adhesion this restricts the transfer of these findings to services using different tubes. Whether a patient remained an inpatient at week four or had been discharged to home care was not recorded either, and the two situations carry different exposures. Only one anatomical site was sampled, so the findings characterise the inner surface of the outer cannula and not the stoma, the outer surface or the inner cannula.⁵ Culture was qualitative and used an enrichment medium, so the 100% positivity rate is a low-threshold determination and should not be read as evidence of heavy colonisation in every patient. The biofilm assay lacked a positive control strain, the number of negative-control wells was not recorded, and the dispersion across replicate wells was not retained, so neither threshold stability nor measurement reproducibility can be quantified.²³ Finally, the classification of the single isolate whose

optical density lay 0.001 units below its plate threshold should be regarded as indeterminate rather than negative, since replicate dispersion is unavailable. Prior antibiotic exposure warrants separate statement rather than inclusion in a list. It is simultaneously the strongest determinant of respiratory flora in hospitalised patients, the strongest driver of resistance selection, and the variable most likely to explain the species distribution reported here, and it was not recorded. Its omission has a non-obvious consequence: recent systemic antibiotics would be expected to suppress planktonic recovery while selecting for tolerant, biofilm-associated organisms, so the omission may bias the species distribution and the biofilm proportion in opposite directions.¹⁰ It also affects the interpretation of the indication variable, because patients reaching week four after prolonged intubation will in most services have had substantially greater antibiotic exposure than those tracheostomised for upper-airway obstruction; the indication variable is therefore in part a proxy for antibiotic exposure, and the null association in Table 4 must be read in that light. Two features of the cohort bound its generalisability further. The near-even split between upper-airway obstruction and prolonged intubation (Table 1) is unusual: in most tertiary centres the great majority of tracheostomies follow prolonged ventilation, and a case mix weighted toward airway obstruction is characteristic of a referral centre serving a wide catchment with limited earlier access to care. And the cohort is entirely adult, with a median age of 56 years and no participant under 26, so the findings do not extend to paediatric tracheostomy, where both the flora and the care model differ.⁷ Recruitment from January to July 2024 spans a substantial part of both the wetter and the drier parts of the West Sumatran year, so the window is not confined to a single season, but 33 patients across seven months does not permit a seasonal analysis.

Future directions

Four studies follow from these findings, in order of priority. The first is a replication with paired antimicrobial susceptibility testing to current CLSI or EUCAST breakpoints, with multidrug resistance defined by the Magiorakos criteria, and with biofilm phenotype and resistance analysed jointly; the published association between the two makes this the study most likely to change prescribing.^{11,34,39} The second is a longitudinal design sampling the same cannula at defined intervals — for example weeks one, two, four and eight — which would convert the present point estimate into an accumulation curve and would allow the optimal exchange interval to be estimated rather than inferred.²⁵ The third is a multicentre Indonesian study spanning several provinces and both wet and dry seasons, which would test whether the Gram-negative predominance observed here is a national pattern or a West Sumatran one, and would feed the national surveillance network directly.^{28,31} The

fourth is an interventional trial of a modified cannula surface or a defined decontamination protocol, with the biofilm index as a quantitative endpoint — a design the present analysis makes feasible by establishing the index's distribution and variability in this population.^{4,35}

Two methodological recommendations follow for any group repeating this work. A single set of negative controls should be run across the whole study wherever the number of isolates allows, and the control value should be reported whatever is done, since without it no reader can reconstruct a classification or test its sensitivity. And the continuous index rather than the dichotomy should carry the analysis, because it is the dichotomy, not the measurement, that proved fragile here.^{23,24}

5. Conclusion

At four weeks after placement, the tracheostomy cannulae of patients at the tertiary otorhinolaryngology referral centre for West Sumatra carried an almost uniformly Gram-negative bacterial flora (93.9% of isolates), dominated by non-fermenting organisms and by *Pseudomonas aeruginosa* in particular, and every specimen yielded growth. Three-quarters of the isolates formed biofilm in vitro and 69.7% did so at moderate or strong intensity, with *Klebsiella pneumoniae* and *Proteus mirabilis* the most consistent producers despite their smaller numbers — a finding that undercuts the intuitive assumption that measures directed at *P. aeruginosa* will address the biofilm problem, since the dominant organism was not the most reliable biofilm former. No patient characteristic, indication for tracheostomy or reported inner-cannula cleaning frequency predicted biofilm production, though the study had only 16–34% power to detect the effects it observed and these null results should not be read as evidence of absence. That a dense, tolerant Gram-negative community is already established on the device by the fourth week supports scheduled tube exchange at approximately that interval and carer education framed around limiting accumulation rather than achieving sterility. These are the first tracheostomy-cannula microbiology data from Sumatra and provide the local evidence that antimicrobial stewardship and national surveillance require. They do not support any empirical antibiotic recommendation, because no susceptibility testing was performed; pairing biofilm phenotyping with susceptibility testing in a multicentre Indonesian cohort is the necessary next step.

Declarations

Ethical approval and consent to participate

Approved by the Health Research Ethics Committee of Dr. M. Djamil Padang Central General Hospital, Indonesia (No. DP.04.03/D.XVI.XI/265/2024). Written informed consent was obtained from all participants.

Conducted in accordance with the Declaration of Helsinki.

Author contributions

AA conceived and supervised the study, provided otorhinolaryngological expertise and is the corresponding author. F was the principal investigator, recruited participants, performed the clinical assessment and collected all specimens. JMR contributed to clinical assessment and data collection. L directed the microbiological work, including culture, MALDI-TOF identification and the tissue-culture-plate biofilm assay, and interpreted the microbiological findings. RR designed the epidemiological framework, performed the statistical analysis and interpreted the public-health implications. All authors contributed to drafting and revising the manuscript, approved the final version, and agree to be accountable for all aspects of the work.

Data availability

The participant-level dataset underlying all figures and tables is reproduced in full within this article and is available from the corresponding author on reasonable request.

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Conflicts of interest

The authors declare no competing interests.

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Generative AI disclosure

The authors declare that no generative artificial intelligence tools were used in the design, analysis, or writing of this manuscript.

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