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Original Article

A critical review of definitions used to describe *Pseudomonas aeruginosa* microbiological status in patients with cystic fibrosis for application in clinical trials

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ABSTRACT

Background: Definition of *Pseudomonas aeruginosa* (Pa) microbiological status is essential for patients' inclusion in clinical trials. The aim of this study was to agree on the definitions of Pa infection status for initial infection, eradication and chronic infection to be used in clinical trials and to propose additional future study areas.

Methods: An exhaustive literature search was performed. The clinimetric properties of different definitions of Pa microbiological status were evaluated.

Results: Historical studies have mostly used culture-based definitions, although some have also involved complementary anti-Pa antibodies. Clinimetric analysis showed great variability in the definitions used, leading to differences in reliability, validity, responsiveness to treatment and correlation with outcome measures.

Use of serology for initial Pa infection and successful Pa eradication introduced a greater level of complexity as antibody tests are not standardised. Moreover, the chronology of the immune response to Pa antigenic determinants was not completely clear.

Chronic Pa infection was characterized by high levels of antibodies and good concordance between culture results and serology.

Conclusions: Microbiological monitoring, regular sampling from the airways and standardization of culture methods remain essential requisites for microbiological definitions. Despite limitations, serology should be incorporated in the definitions of initial infection and eradication used in clinical trials to better classify patients at enrolment, mainly in non-expectorating children. This requires standardization of serological testing.

Abbreviations: ABx, antibiotics; AET, antibiotic eradication therapy; AP, alkaline protease; AUROC, area under the ROC curve; Azithro, azithromycin; AZLI, aztreonam lysine; BAL, bronchoalevolar lavage; Ceft, ceftazidime; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CFU/ml, colony forming units/ml; CIE, Crossed immune electrophoresis; Cip, Ciprofloxacin; clav, clavulanate; Col, colistin; EAT, early antibiotic therapy; Ela, elastase; ELISA, Enzyme-linked immunosorbent assay; ECFS-CTN, European Cystic Fibrosis Society-Clinical Trials Network; EU, ELISA unit; Exo/ExoA/T/S, exotoxin A/T/S; FEV_1% pred, forced expiratory volume % predicted; FVC, forced vital capacity; grp/s, group/s; inh, inhaled; IgG, immunoglobulin G; IQR, interquartile range; IV, intravenous; MAg, multiple antigenic blend; MC, monoclonal antibody; mg, milligrams; mo, month/s; n/no, number; NA, not applicable; neb, nebulized; NIH, National Institutes of Health Score; NPV, negative predictive value; NR, not reported; NS, not significant; OP, oropharyngeal; OR, odds ratio; p, *p*-value; Pa, *Pseudomonas aeruginosa* ; popn, population; PopB, PopB protein; PopD, PopD protein; PPV, positive predictive value; PS/PI, pancreatic sufficient/pancreatic insufficient; pred, predicted; resp., respiratory; rhDNase therapy, Recombinant human deoxyribonuclease therapy; RIA, radioimmunoassay; ROC, receiver operating curve; Rx, treatment; SD, standard deviation; SOP, standard operating procedure; St-Ag, standard antigen; Tic Clav, ticarcillin clavulanate; TIS/TSI, tobramycin inhalation solution; TIV, tobramycin by IV administration; tobi, tobramycin; WBC, white blood cell count; wk./s, week/s; VC, vital capacity; vs, versus; yr/s, year/s; −ve, negative; +ve, positive.

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1. Introduction

Pseudomonas aeruginosa (Pa) is the most important pathogen in progressive lung disease in patients with cystic fibrosis (CF) [1]. Following initial infection, in the absence of prompt antibiotic treatment, chronic infection is likely to develop, resulting in severe inflammation that leads to progressive lung damage and deterioration in the clinical condition of the patient. Determination of the stage of infection, e.g., initial or chronic Pa infection, as well as successful Pa eradication, is essential for standard of care as it dictates antibiotic strategy. Initial Pa infection usually occurs in young patients; it is typically asymptomatic and caused by strains which show susceptibility to anti-pseudomonal agents [2]. Recent studies have emphasized the importance of promptly initiating antibiotic eradication treatment (AET) as once chronic infection is established, it is rarely possible to eradicate Pa [3,4]. Chronic Pa infection is associated with a steeper deterioration in lung function, frequent pulmonary exacerbations and a shorter median survival [5–8]. In clinically stable patients, chronic Pa infection is usually managed by administering long-term courses of inhaled antibiotics and/or azithromycin [9]. Definition of Pa infection status relies on standardised procedures for respiratory sampling, specialised laboratory expertise in recognising CF pathogens and careful microbiological follow-up [10,11]. As detection of Pa is very dependent on sputum sampling and culture techniques, other biomarkers of infection have been considered. Anti-Pa antibodies have long been used to support microbiological diagnosis both in the initial stage of infection, and in chronicity. Commercial kits are easily available in Europe and previous studies have measured antibody responses to individual Pa virulence factors or to multiple antigens from 17 of the most common serotypes (St-Ag:1–17) [12]. However, the chronology of the immune response to Pa antigenic determinants is only partially known and there is no consensus regarding the best performing kit.

There is great interest in the CF community in developing standardization for clinical trial outcome measures. The European Cystic Fibrosis Society Clinical Trials Network (ECFS-CTN) has established a Standardization Committee to undertake a rigorous evaluation of clinical trial endpoint [1,13]. This article summarises the work of the Standardization Committee's Microbiology Group on definitions of Pa infection status for application in clinical trials. It is crucial for anti-infective clinical trials to confirm an individual patient's eligibility and assessment outcome criteria. Historically, trials of antimicrobials in CF have been based on different definitions of Pa infection status [4,14]. The aim of this document is to offer guidance for investigators, pharmaceutical companies, and regulatory authorities when planning trials in CF patients including Pa microbiological status as an outcome. After critical review of the literature in support of definitions for Pa infection status, the group gained consensus on the definitions of initial Pa infection, eradication and chronic infection and proposed additional future study directions.

2. Methods

An exhaustive literature search was conducted in PUBMED using the following search criteria: Initial/first lifetime/early/new Pa colonisation/infection, Pa eradication, anti-Pa antibodies, chronic Pa infection AND cystic fibrosis. A bibliography search was also conducted of all included articles and relevant reviews. The papers were checked in order to verify that definitions regarding Pa microbiological status were indeed reported.

The clinimetric properties of Pa microbiological status definitions were assessed, including their reliability, validity, responsiveness to treatment, reference values, and correlation with other outcome measures. Definitions of each clinimetric property are presented in Supplementary Table S1 [15].

The expert panel searched for a consensus on definitions regarding Pa microbiological status and the use of anti-Pa antibodies, including the performance of tests available, the availability of standardised procedures, training requirements, their cost, the equipment/space required, and their applicability to different age groups.

The levels of evidence that underlies recommendations in this document is based upon Scottish Intercollegiate Guidelines Network [16].

3. Results

3.1. Definitions found in literature

3.1.1. Definition of initial (first lifetime/early/new) Pa infection

Table 1 describes the different definitions used for first lifetime or positive Pa culture after a stated time scale in studies aiming for Pa eradication. This includes first lifetime documented culture positive for Pa [17–20] or positive Pa culture following a negative Pa timescale for: 6 months [21–23], 1 year [24–29] and 2 years [30–32].

Other differences among the studies included the type/combination of samples collected sputum versus oropharyngeal (OP) swabs versus BAL [21,23,25,33–37] versus laryngeal suction [28,38]. Most of the studies combined different measurements which further complicates interpretation of the data. The studies with bronchoalveolar lavage (BAL) focused on infants until able to expectorate sputum and were performed at diagnosis of CF (6 to 12 wks) and annually thereafter [35,37]; or on patients with OP culture positive for Pa [36].

The most sophisticated studies included verification of positive results from previous clinical cultures with further cultures during a screening period in combination with anti-Pa antibodies [27,29,31]. Ratjen [27] also used positive antibody titres for any of the three Pa exoproducts as an exclusion criterion [27].

3.1.2. Definition of Pa eradication

Definitions of successful eradication varied according to the studies in terms of time-point for sampling (end of treatment versus a time point after treatment) (Table 2). The Pa free interval varied from (i) 3 months [17,35] (ii) 6 months with 3 successive negative cultures [18,22]; (iii) 12 months [34,37,39,40] with 2 negative consecutive respiratory cultures [26]; (iv) a variable follow-up timescale e.g. free of Pa at 28, 56 and 84 days [33] or weeks 4,8,16 and 28 [31]. Other definitions included Pa free interval 1 month after end of treatment [24,27] or negative BAL Pa culture at day 56, 84 or 112 after treatment [36].

As stated above, some definitions of eradication varied according to the number of follow-up samples collected at various timepoints (single culture versus several cultures over 6 months or more). In the case of several samples the strategy was very variable: e.g. quarterly follow up samples for the study duration of up to 2 years [39]. Studies also varied by type of samples collected (sputum versus OP swab versus BAL versus laryngeal suction), the use of anti-Pa antibody testing and the time to Pa recurrence or chronic Pa. As for initial infection, within individual papers there is often no clear discrimination between sample types which renders studies difficult to analyze.

Several studies incorporated the use of serology in the definition of eradication and/or study outcomes. Tiddens [31] determined baseline anti-Pa antibodies (negative/positive) [31]; whilst Proesmans [21] assessed Pa antibodies (ELISA St-Ag 1–17) at one year follow up [21] and Gibson [36] assessed serology at end of treatment [36], follow-up BAL and day 168 visits. Ratjen [27] used

Table 1
Studies assessing first lifetime or positive *Pseudomonas aeruginosa* (*Pa*) culture after stated timescale in studies aiming for *Pa* eradication.

Author year	Patients (n)	Age (yrs) Mean (SD) Or (median) where stated	Sample origin (OP swab/sputum/BAL)	Treatment	Serology used in definition of early infection	Serology used as study inclusion/exclusion criteria or endpoint?
First lifetime documented culture positive for Pa						
Mayer-Hamblett [30]	221	6 mo to 18 yrs	OP swab or lower resp. tract culture	Oral Azithromycin and TIS	No	No
Schelstraete [17]	41	6mo to 30 yrs	Naso-pharyngeal aspirates or sputum	Oral Cip and TIS or Col	Yes	No
Taccetti [18]	58	8.9 (±6.6)	Sputum	Inh Col and oral Cip	Yes	Yes All patients included had −ve serum antibody titres against Pa and precipitating anti-Pa antibodies <2
Ratjen [19]	15	9 (+4.6)	Swab or sputum	TIS	No	Yes Exclusion criteria: if patients had previous throat swabs or sputum positive for Pa or positive serum antibodies against Pa Endpoint: Absence of infection confirmed by −ve serum antibody titres against Pa
Valerius [20]	14	8.5 (range 36–228 mo)	Sputum	Inh Col and oral Cip	No	Yes: study endpoint: when colonisation with Pa became chronic and/or precipitins ≥2
2 year negative Pa timescale >2 negative cultures/year						
Mayer-Hamblett [30]	221	6 mo to 18 yrs	OP swab or lower resp. tract culture	Oral Azithromycin and TIS	No	No
Tiddens [31]	105	6.2 (±4.7)	Sputum or swab	AZLI (inh)	No	Stated as other endpoint of interest: Pa specific serum antibodies (Exo A, Ela, AP)
Minimum of 1 documented negative Pa culture per year						
Treggiarri [32]	304	1 to 12	Sputum or swab	TIS with either oral Cip or placebo	No	No
1 year negative Pa timescale >3 Pa negative cultures in previous 1 year (previously Pa positive)						
Stanojevic [25]	65	7.4 (median) range 3.2 to 10.3	Swab, sputum or BAL	TIS	No	No
Hansen [28]	99	4.8 (range 0.3 to 28.1)	Sputum or *endo*-laryngeal suction	Inh Col and oral Cip	No	Yes Inclusion criteria: All patients included had precipitins <2. Study endpoint: 10/12 patients developing chronic infection (Copenhagen definition)
Wieseman [29]	11	9.8 (+8.3)	Sputum or swab	TIS or placebo	No	Yes Inclusion criteria: Pa + ve respiratory cultures in at least 2 of 3 specimens and seronegative for Pa antibodies(Exo A, Ela, AP)
At least four documented negative cultures (or up to 2 years with 4 negative cultures in this time period in the absence of anti-Pa treatment):						
Ratjen [24]	51	39 mo (median) (range 6–82)	Sputum or swab	TIS	No	No
Ratjen [27]	88	6mo to >18 yr	Sputum or swab	TIS	No	No
Amin [26]	116	7.5 (±1.9)	Sputum	TIS	No	No
6 month negative Pa timescale						
Proesmans [21]	58	9 (median) range 4.7–13.1	Swab, sputum or BAL	TIS or Col plus oral Cip	No	Yes Study endpoint: Pa antibodies determined at 1 year after study entry (ELISA St Ag 1–17)

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Table 1 (*continued*)

Author year	Patients (n)	Age (yrs) Mean (SD) Or (median) where stated	Sample origin (OP swab/sputum/BAL)	Treatment	Serology used in definition of early infection	Serology used as study inclusion/exclusion criteria or endpoint?
Taccetti [22]	105 118	7.45 (IQR:1 to 25.5) 7.64 (IOR:1 to 35.2)	Sputum or swab	TIS and oral Cip vs Inh Col and oral Cip	No	No
Frederiksen [23]	48	7.4 (range 0.9–18.4)	Sputum or swab or nasopharyngeal aspirate or BAL	Inh Col and oral Cip	Yes Intermittent colonisation: presence of Pa in sputum at least once in association with normal values of Pa precipitins (0–1).	Yes Endpoint was chronic Pa infection defined as persistent presence of Pa in sputum for at least 6 months or less when combined with presence of ≥2 precipitins.
Presence of Pa in BAL culture: 1st lifetime Pa isolation:						
Douglas [35]	33	2.5 (range 3–71 months)	BAL at diagnosis (6 to 12 wks) and annually until able to expectorate sputum	IV Tobi and tic clav OR Ceft, followed by TIS and oral Cip	No	No
Nixon [37]	24	3.7(±2.1) (range 0.3–7)	BAL at diagnosis (6 to 12 wks) and annually until able to expectorate sputum	Tic clav and Tobi, followed by oral Cip and/or neb Tobi	No	No
Other definitions First/New Pa isolation (not stated if first lifetime isolation):						
Blanchard [33]	128	9 (median) IQR 6.8–12.3	Sputum or swab or BAL	Step 1: TIS Step 2: TIS Step 3: IV Ceft and Tobi plus TIS	No	No
Kenny [40]	20	27 (median) range 18–81 yrs	Sputum	Oral Cip/neb Col IV ABx therapy Other: TIS and oral Cip; Oral Cip Neb Col	No	No
Isolation of mucoid Pa: at least 1 positive Pa sample in 9-year retrospective study period						
McPherson [34]	116	<16 yrs	Sputum or swab or nasopharyngeal aspirate or BAL	Inh Col and gentamicin	No	No
First Pa isolation: OP culture positive for Pa within 2 weeks to 12 months before screening (not stated if first lifetime isolation)						
Gibson [36]	28	>6mo–<6 yrs.	OP culture positive for Pa then eligible for BAL	TIS	No	Yes (ExoA serology assessed at baseline)
Initial isolation: presence of Pa in sputum and fewer than 2 precipitin antibodies against Pa (not stated if first lifetime isolation)						
Munck [38]	19	5.0 (+4.0) (range 3mo–14 yrs)	Sputum or laryngeal suction	IV Tobi and neb Col	Yes Colonisation: presence of Pain sputum and <2 precipitins against Pa	No

ABx: antibiotics; AP: alkaline protease; AZLI: aztreonam lysine; BAL: bronchoalveolar lavage; ceft: ceftazidime; Cip: Ciprofloxacin; clav: clavulanate; Col: colistin; Ela: elastase, ELISA/St-Ag1–17: Enzyme-linked immunosorbent assay/standard antigens 1–17; Exo/ExoA: exotoxin A; FEV_1% pred: forced expiratory volume % predicted; grp/s: group/s; inh: inhaled; IQR: interquartile range; IV: intravenous; mg: milligrams; mo: month/s; n: number; neb: nebulized; NS: not significant; OP: oropharyngeal; p: p-value; Pa: *Pseudomonas aeruginosa; resp.: respiratory*; Rx: treatment; SD: standard deviation; Tic Clav: ticarcillin Clavulanate; TIS: inhaled tobramycin solution; Tobi: tobramycin; wk./s: week/s; vs: versus; yr/s: year/s.

positive antibody titres as an exclusion criterion [27] and assessed serology at baseline and 1 year follow up [41]; Taccetti [18] employed negative titres within a 6-month period post treatment [18].

3.1.3. Definition of chronic Pa infection

Definitions of chronic Pa infection previously used in trials of maintenance therapy were assessed by culture time point and sample origin. Data is outlined in Table 3. Similar to initial Pa acquisition and eradication, studies differed widely in the criteria used to define chronic Pa infection. This included a wide range of timeframes required for sputum specimen positive for Pa: from 3 months [42]; six months prior to screening [43–49], 12 months [9,49–55]; 18 months [57] and finally 24 months prior to screening [58]. Five other studies did not specify a timeframe. The majority of studies did not specify the total number or type of samples required apart from the following specific definitions: 3 positive specimens within 2 years prior to screening and 1 within 3 months prior to screening [58]; 2 positive cultures within 12 months prior to screening (1 within the previous 3 months) or positive at screen-

Table 2
Definitions of *Pa* eradication in studies assessing the efficacy of early eradication treatment.

Author year	Patients (n)	Age (yrs) mean (SD) or median where stated	Sample Origin (OP swab/sputum/BAL)	Treatment	Culture time-points	Follow up duration	Efficacy of treatment (% eradication)	Eradicated popn vs non-eradicated popn	Serology used in definition of eradication?
12 Months									
Free of Pa for 12 months at quarterly assessment (all quarterly cultures free of Pa)									
Mayer Hamblett [39]	304	7.6(4.1)	Sputum or swab	TIS Oral Cip Oral placebo	Quarterly for 1 to 2 yr study period	5 yrs	69	Eradicated cohort reduced risk of developing chronic Pa by 74% ($p < 0.001$) Eradicated cohort 50 to 60% less ABx and macrolide usage ($p < 0.001$)	No
Kenny [40]	20	27(median) 18–81	Sputum	Oral Cip/neb Col	Bimonthly intervals pre-Pa isolation then median interval of 24 days post isolation until eradication	1 yr	23.9	NS between grps	No
McPherson [34]	116	10.3	Sputum or swab or nasopharyngeal aspirate or BAL		Reported as earliest culture available until last available result over 9-year period	9 yrs	81	Eradicated cohort mean age at clearance lower ($p = 0.049$)	No
Nixon [37]	24	3.7 ± 2.1 (0.3–7)	BAL at diagnosis (6 to 12 wks) annually thereafter until able to expectorate	IV Tic/Clav & Tobi followed by oral Cip and/or TIS	Initial then annual for 2 years	7 yrs	25	Non-eradicated cohort: Lower NIH score ($p < 0.01$) Higher no. days hospitalized ($p < 0.01$)	No
Free of Pa for 2 negative consecutive respiratory cultures, measured quarterly for 1 year of follow up									
Amin [26]	116	7.1 ± 1.9	Sputum	TIS	Quarterly follow-up	5 to 7 yrs	52	NS between grps	No
6 Months									
Pa eradication: 3 successive negative cultures in 6mo									
Taccetti [22]	105 118	7.45 (IQR:1 to 25.5) 7.64 (IOR:1 to 35.2)	Sputum or swab	Inh Col plus oral Cip OR TIS plus oral Cip	Month 2,4,6	16 mo	63 (Cip/Col) 65 (Cip/Tobi)	FEV_1% mean relative change from baseline 2.15% (+8.50) in grp A and 4.55% (+11.54) in grp B ($p = 0.18$)	No
Taccetti [18]	58	8.9 ± 6.6	Sputum	Inh Col plus oral Cip	Quarterly	Not stated	80 Pa isolates from Rx grp more sensitive to Pa ABx ($p < 0.05$)	Average FEV_1% decline sig lower in early treated-patients vs chronically-infected ($p < 0.05$)	Yes Serology used in definition of eradication: −ve titres ExoA and precipitins <2 within 6-month period post Rx
3 Months									
Schelstraete [17]	41	6mo to 30 yrs	Naso-pharyngeal aspirates or sputum	Oral Cip plus TIS or Oral Cip plus Col	Monthly during eradication period then every 3 months post eradication treatment period	50 mo median	83 47 at median follow up at 39 mo Genotypes of 1st and subsequent Pa isolates: chronic colonised patients identical genotypes 1st and 2nd isolates ($p < 0.0001$)	Pa free period between 1st ever and 2nd Pa isolation: chronic colonised patients shorter time to 2nd isolate ($p < 0.05$)	No
Douglas [35]	33	2.5 (3–71) mo	BAL at diagnosis (6 to 12 wks) and annually thereafter until able to expectorate sputum	IV Tobi plus Tic/Clav OR IV Tobi plus Ceft followed by TIS and oral Cip	Annually, 12 months prior, at Pa detection and 12 months post eradication	19 mo median	88.5	Decrease pulmonary inflammation $p < 0.05$ (neutrophils % total cell count; IL-1B); $p < 0.001$ (neutrophils log, NE, IL-6)	No

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Table 2 (*continued*)

Author year	Patients (n)	Age (yrs) mean (SD) or median where stated	Sample Origin (OP swab/sputum/BAL)	Treatment	Culture time-points	Follow up duration	Efficacy of treatment (% eradication)	Eradicated popn vs non-eradicated popn	Serology used in definition of eradication?
Variable timescale: Free of Pa at 28, 56, 84 days									
Blanchard [33]	128	9 median IQR 6.8–12.3	Sputum or swab	TIS (step 1) TIS (step2) IV Ceft and Tobi and TIS (step 3)	Day 28,56,84	5 yrs	76.9 at end step 1 (28 days TIS) 33.3 at end step 2 (additional 28 days TIS) 87.1 at all time points	PS increased risk of failure to eradicate BMI z score lower in PI	No
Free of Pa at weeks 4,8,16 and 28									
Tiddens [31]	105	6.2 ± 4.7	Sputum or swab	AZLI	Week 4,8,16,28	24 wks	89end of Rx 75 4 weeks post Rx 58 at all time points	Patients –ve for Pa maintained mean baseline FEV_1% pred throughout 24-week follow-up (n = 25)	Yes antibodies as endpoint of interest: AP, Ela, ExoA Titres: –ve: <500 Borderline: >500 to <1250 +ve: >1250 to <10,000
Free of Pa upon completion of treatment									
Stanojevic [25]	65	7.4	Swab, sputum or BAL	TIS	83 days median (TIS 80 mg grp) 21.5 days median (TIS 300 mg grp)	1 yr	89 (80 mg grp) 89 (300 mg grp)	Pa recurrence in 12 months post Rx: female, age, PS patients, lower lung function, lower BMI $p < 0.001$	No
Free of Pa upon completion of treatment as primary outcome at 28 days (TIS) or 3 months (CC), and as secondary outcome at 6 months									
Proesmans [21]	58	4.6–14.6 9 median (IQR 4.7–13.1)	Swab, sputum or BAL	Cip-TIS or Inh Col plus oral Cip	consecutive months	2 yrs	79.3 Cip-TIS 89.7 Cip-Col	NS between grps	Yes St-Ag 1–17 antibodies determined at 1 year
1 month after the end of treatment									
Ratjen [27]	88	6mths to >18 yrs	OP swabs/sputum	TIS	Month 3,12,27	27 mo	93 TIS/28-day 1mo post Rx 92 TIS/56-day 1mo post Rx 66 TIS 28 day at mo 27 69 TIS 56 day at mo 27	NS	Yes Patients excluded if AP, Ela, ExoA titres were >1000
Ratjen 2018	51	39 mo median (range 6–82)	OP swabs/sputum	TIS	Day 29, 63, 91 for persistent eradication	273 days (median) Range 65–379 days	84.6 at day 29	NS	No
At time of follow up BAL (day 56, 84 or 112)									
Gibson [36]	28	2.4 + 2.0	BAL		Day 30,56,84,112,168	168-day BAL follow up	74	-ve serology at baseline higher proportion of eradication $p < 0.04$ Inflammatory markers: TSI Rx associated with reduced neutrophilic inflammation	Yes ExoA +ve >1:200 or higher Non-mucoid Pa at baseline and/or ExoA seronegativity associated with higher rates of eradication
Negative sequential respiratory cultures									
Ratjen [19]	15	9 + 4.6	Swab	TIS	Month 3,6,9,12	2 yrs.	14/15 at 1 year	NS	Yes AP, Ela, ExoA Titres not stated. Serology -ve titres for Pa
Pa eradication: absence of Pa or presumed presence at a level below the detection limit any time point									
Munck [38]	19	5.0 + 4.0 (3 m–14 yrs)	Sputum or laryngeal suction	IV ceft or imipenem with Tobi, inh Col	Bimonthly or more often if clinically indicated	3 yrs	100 74	NS	Yes All patients had <2 precipitins after first and second Pa isolation

ABx: antibiotic/s; AP: alkaline protease; AZLI: aztreonam for inhalation solution; BAL: bronchoalveolar lavage; BMI z score: body mass index z score; Ceft: ceftazidime; CIE: Crossed immune electrophoresis; Cip: Ciprofloxacin; Col: Colistin; Ela: elastase; ELISA: Enzyme-linked immunosorbent assay; Exo A: exotoxin A; EAT: early antibiotic therapy; FEV_1% pred: forced expiratory volume predicted; grp/s: group/s; inh: inhaled; IL-1B: Interleukin 1beta; IL-6: Interleukin 6; IQR: interquartile range; IV: intravenous; mg: milligrams; mo: month/s; Pa: *Pseudomonas aeruginosa*; n/no: number; NE: neutrophil elastase; NIH: National Institutes of Health Score; NR: not reported; NS: not significant; OP: oropharyngeal; p: p-value; Pa: *Pseudomonas aeruginosa*; popn: population; pred: predicted; PS/PI: pancreatic sufficient/pancreatic insufficient; resp.: respiratory; Rx: treatment; SD: standard deviation; sig: significant; St-Ag: standard antigen; Tic Clav: ticarcillin clavulanate; Cip: oral ciprofloxacin; Tic Clav: ticarcillin Clavulanate; TIS: tobramycin inhalation solution; tobi: tobramycin; –ve: negative; +ve: positive; vs: versus; wk./s: week/s; yr/s: year/s.

Table 3
Studies defining chronic Pa infection: sample origin and culture time-point.

First author/year	Patients (n)	Age (yrs) mean (SD)	Sample origin (OP swab/sputum/BAL)	Culture time-points (days) [unless otherwise stated]
24 Months				
Sputum specimen positive for Pa within 24 months prior to screening (3 positive cultures) and 1 positive culture within 3 months prior to screening				
Okusanya [58]	105	20(6.68)	Sputum	Baseline,14,28
18 Months				
Sputum specimen positive for Pa within 18 months prior to screening and positive at screening				
Geller [57]	151	28.7	Sputum	Baseline,28
12 Months				
Sputum specimen positive for Pa within 12 months prior to screening and positive at screening				
Flume [50]	330	28.8 (10.9) 29.4 (10.3) placebo	OP or sputum	14,28,42,56
Elborn [51]	282	28.8(10.94) 28.1(8.96)	OP or sputum	Baseline, 28,56,84,112,140,168
Okusanya [54]	24	23.7(6.96)	Sputum	Screening −14,0,1,7,14
Saiman [9]	185	20.2(7.9)	OP or sputum	Screening,168, 252,336
Sputum specimen positive for Pa in 2 cultures within 12 months prior to screening (1 within previous 3 months) or positive at screening				
Trapnell [52]	119	35(10.9) 31(8.8) placebo	OP or sputum	Baseline,14,28,42,56
Wainwright [53]	157	31(10.2) 18.9(9.1) placebo	OP or sputum	Baseline,28,42
Retsch-Bogart [55]	138	19.5(9.1) AZLI 31.7(11.74) placebo 27.4(7.54) AZLI	Sputum	Baseline,14,28
Hodson [49]	115	21.3 ± 9.6 TNS 20.1 ± 9.4 Colistin	Sputum	Screening (week −2), baseline (week 0), week 4
Sputum specimen positive for Pa (1 historical OP sample) within 12 months prior to screening (2 weeks to 12 months prior) and positive baseline BAL culture at screening				
Gibson [56]	21	4.0(1.5) TIS 3.7(1.6) placebo	BAL	Baseline,28
6 Months				
Sputum specimen positive for Pa within 6 months prior to screening and positive at screening				
Konstan et al., [43] (JCF)	517	26(11.4) TIP 25(10.2) TIS	OP or sputum	Baseline,28,56,84,112,140,168
Konstan et al., [44] (Ped Pulm)	95	13.4(4.42) TIP 13.2(3.91) placebo	OP or sputum	Baseline,28,56,84,112,140
Herrmann [45]	5	27(9)	Sputum	Baseline,30
Nasr [46]	32	11.81(7.46) TSI 15.86(7.25) placebo	OP	Baseline,28 (PFTs end of study, not cultures)
Chuchalin [47]	247	14.8(5.7) TSI 14.7(6.6) placebo	Sputum or IS/sputum	Baseline,14,28,56,84,112,140,168
Lenoir [48]	59	11.0(5.0) TSI 14.2(5.5) placebo	IS/sputum	Baseline,28,56
Hodson [59]	20	15–42 range	Sputum	Baseline, monthly for 12 mo
6 consecutive monthly sputum samples with growth of Pa or in case of shorter colonisation, rise in specific, precipitating antibodies against *P. aeruginosa* to at least 2				
Hansen [28]	99	4.8(range 0.3 to 28.1)	OP or sputum	Screening, monthly until establishment of chronic infection
3 Months				
Sputum specimen positive for Pa within 3 months prior to screening				
Assael [42]	268	25.8(9.1) AZLI 25.1(9.0) TNS	Sputum	Baseline,28,84,140
Variable sample/time-point parameters				
Sputum specimen positive for Pa in 2 prior samples				
Murphy [104]	181	9.9(2.4) control 10.2(2.7) TIS	OP or sputum	Baseline,56,140,224,308,492
Sputum specimen positive for Pa in 1 prior sample				
Burns [105]	520	>6 years	OP or sputum	Baseline,140 (168 for TSI)
Sputum specimen positive for Pa at screening visit				
McCoy [106]	211	27.9(10.65) placebo 26.5(10.50) AZLI 24.1(7.5) AZLI	OP or sputum	Baseline,14,28,42
Sputum specimen positive for Pa				
Ramsey [107]	464	20.8(9.5) TIS 20.6(10) placebo	Sputum	Baseline,12,28,42,56,84,112,140,168
Ramsey [108]	71	17.7(1.25) TIS 16.6(1.24) placebo	Sputum	Baseline,14,28,56,84 (TIS) Baseline,14,21,28,35 (placebo)

AZLI: aztreonam lysine; BAL: bronchoalveolar lavage; n: number; mo: month/s; OP: oropharyngeal; Pa: Pseudomonas aeruginosa; Rx: treatment; SD: standard deviation; TIS/TSI/TIP: tobramycin inhaled solution/powder; TNS: tobramycin nebulised solution; wk./s: week/s; yr/s: year/s.

ing [49,52,53,55]. Pa was confirmed using repeated culture as part of the screening or baseline measurements in most of the studies [9,43–51,54,56,58,59]. Only one study incorporated anti-Pa antibodies, defining chronic Pa infection as a continuous presence of Pa in the lungs for more than 6 months and/or the presence of more than 2 precipitating Pa antibodies [47].

Due to the difficulties in defining chronic Pa infection and hence the identification of patients most at risk of progressive lung disease, recent research aims to redefine chronic infection in CF either by considering the emergence of mucoidity or by using molecular methods. Heltshe [60] demonstrated that the majority of children developed mucoid infection prior to meeting their accepted definition of chronic infection (at least 3 yearly quarters Pa positive in the preceding year) [60]. Boutin [61] showed that Pa abundance, measured by quantitative polymerase chain reaction (qPCR), was more discriminatory than mucoidity to distinguish chronic from intermittent Pa strains [61].

3.2. Assessment of the clinimetric properties of microbiological definitions of Pa status based solely on cultures

3.2.1. Feasibility

The microbiological definitions of Pa status require a review of historical culture results. This requires all ECFS-CTN centres to adhere to agreed sampling frequencies, sample types and laboratory processes *prior to* trial enrolment. Therefore, these centres must agree to adhere to a set of minimum standards during their routine clinical practice. Culture of respiratory samples is easy to perform and is now supported by the use of ECFS-CTN Standard Operating Procedures (ECFS-CTN SOP Optimised Isolation and Identification of Pa; ECFS-CTN SOP Frequency and Methods of Respiratory Sampling; ECFS CTN SOP Sputum Induction; ECFS-CTN SOP Inflammatory Mediators in CF Patients Bronchoalveolar Lavage Through a Flexible Bronchoscope) which recommend sampling on a quarterly basis of sputum, or induced sputum in non-expectorating patients [62–65]. The feasibility of sampling will be variable depending on patient factors. Older children and adults who expectorate sputum will be easy to sample. It may be difficult to collect sputum from younger children unable to expectorate. As OP swabs or laryngeal suction lack optimal sensitivity, patients with early infection may be missed in clinical trials. Recent studies utilizing molecular methods such as qPCR may prove to be beneficial in the early detection of lower airway Pa infection using OP swabs in a non-expectorating paediatric population [61]. Importantly, recent studies highlight the utility of sputum induction as a surrogate for BAL for paediatric lower airway pathogen assessment in symptomatic children [66].

3.2.2. Risk

There will be additional clinical and enrolment risks should invasive sampling be required (e.g. via two-lobe BAL).

3.2.3. Responsiveness

The outcome measure of 'successful Pa eradication' is required and conversion from positive to negative Pa culture results is optimal to monitor the efficacy of the intervention (Table 3).

Eradication is associated with less antibiotic therapy [39], reduced lung function decline [18], and reduced risk of worse lung function [25]. In a 7-year prospective study by Nixon [37] Pa infection was associated with increased time of hospitalization, and higher rates of rhDNase therapy in survivors [37]. Effect on nutritional status was controversial, with no change [21,27]; whereas 1 study demonstrated a significant increase in mean ideal weight for height of 3.5% ($p < 0.001$) [67]. A possible side effect of EAT could be the emergence of new pathogens in the airways as reported in 2 studies [22,32].

3.2.4. Cost

The costs of non-invasive sampling are minimal. These will increase if either induced sputum or bronchoscopy is required. Culture costs should be minimal, although these would be increased if Pa quantitative counts are needed, if the trial protocol requires repeated cultures over a prolonged period of time or if additional transport costs were incurred (e.g. to a central laboratory).

3.3. Assessment of the clinimetric properties of microbiological definitions of Pa status based on sputum culture and anti-Pa antibodies

3.3.1. Validity

3.3.1.1. Concurrent validity. In the early stages of Pa infection, the use of anti-Pa antibodies can be justified because of the low sensitivity of OP swabs obtained from non-expectorating patients. Anti-Pa serology, in the initial phase of Pa infection (Tables 1 and 2), could be considered *an adjunct to, rather than a replacement for* cultures. At present, no clinical decisions regarding patient treatment are based on anti-Pa serology alone.

Chronic Pa infection is characterized by high levels of anti-Pa antibodies and good concordance between culture results and serology (Table 4). Clear cut-offs have only been well defined for chronic Pa infection [41,68–70]. Cut-offs for 'significant' rises in Pa antibodies in the chronic phase of Pa infection would need to be defined.

3.3.2. Predictive validity

3.3.2.1. Diagnosis of early infection. A substantial overlap has been described between anti-Pa antibody titres in those patients who did subsequently acquire Pa and in those patients who did not [12]. An immune response to Pa can occur earlier than culture positivity [41,68,71]. These data highlight the need for anti-Pa antibodies as a *complementary* test to cultures in the early stages of infection. Moreover, elevated levels of anti Pa antibodies (ExoA and AP) prior to AET may be associated with higher risk of Pa re-isolation during follow-up [72] (Table 5).

3.3.2.2. Predictive value of anti-Pa titres following AET. There is a slight discordance between culture results and serology in the post-treatment period. "Culture decline" (i.e. culture conversion from Pa-positive to Pa-negative) shows a different trend in comparison with "serology decline" (i.e. conversion from Pa-antibody positive to Pa-antibody negative) [71].

Nevertheless, a decline in anti-Pa antibody titre over one year can predict success of AET [73]. Similarly, an increase in titres over time is described in AET failure [41,74,75] or evolution towards chronic infection [74] (see Table 5).

3.3.2.3. Cost. The costs of commercial kits may vary from country to country as will laboratory overheads (e.g. staff costs, utilities, etc.). A standardised method of Pa antibody testing would be required. This would either be the same kit/method in several labs or one test in a single central laboratory. The latter would then require additional costs for transport of serum samples.

4. Discussion

Analysis of Pa microbiological status used in literature on patients with initial or chronic Pa infection, as well as eradication clinical trials, has revealed great variability of definitions.

4.1. Respiratory sampling

The methods used for the definition of Pa microbiological status of CF patients, may be subject to various technical issues. Diagno-

Table 4
Anti-Pa antibodies at chronic Pa infection.

First author year	Patients (n)	Age range (yrs)	Pa time sampling	Method	Titre cutoff	Antigen	% sens	% spec	PPV	NPV	Antibodies assessed during Rx and at follow-up?	Evidence of seropositivity/increase in titres
Daines [12]	261	0.0–12.0	1st lifetime	ELISA	<100: −ve >100: +ve	ExoA	AT CHRONIC INFECTION: (6 mo)				Baseline and follow up (annual samples over 3 yr period)	Yes at 6 mo No at 12 mo
						AP	39.1	77.6	47.8	70.7		
						Ela	24.9	74.8	34.1	65.4		
						3/3	23.7	84.1	44.0	67.7		
							10.7	95.6	56.2	67.0		
						ExoA	AT CHRONIC INFECTION (12 mo)					
						AP	35.6	77.6	56.4	59.7		
						Ela	23	74.8	42.6	54.4		
						3/3	19.2	84.1	49.5	56.1		
							8.4	95.6	61.1	56.2		
Kappler [73]	58	0.4–25.9	One yr post first detection of Pa	ELISA	-ve <1:500 +ve >1:500	AP	43	94	82	71	Antibodies one yr post Pa detection	Pa antibodies predict success of early eradication Rx at 1 yr
						Ela	52	90	79	74		
						ExoA	48	97	91	74		
						cumulative	71	84	75	82		
Dōgru [109]	90	8–26.3	4 grps: negative Pa; intermittent; chronic; mucoid	ELISA	NR	AP	84	40	100	77.8	1st visit and 2 yr follow up	No at 2 year follow up
						Ela	80	44.4	100	80.0		
						ExoA	80	43.3	100	78.4		
Anstead [72]	303	1.0–12.0	1st lifetime	ELISA	for E 15 ELISA: <1:500: −ve >1:500: +ve	AP3	Pooled across 4 time-points				4 time-points: wks 0,22,46 and 70	Seropositivity to AP and ExoA sig assoc. with increased risk of recurrent Pa during 60-week post eradication follow up ($p = 0–003$, $p = 0.001$)
						Ela	45	81	38	85		
						ExoA	52	78	38	86		
						MC antigens[c]	67	58	29	87		
						PopB +ExoS	69	65	33	89		
						PAO1	48	83	42	86		
							60	72	35	88		
Cruz [110]	27	2.5–16.8	N = 11 initial Pa −ve (N = 16 chronic Pa)	Western Blot	[d]	ExoS ExoT PopB PopD	NR	NR	NR	NR	Assessed at 4 time-points	Yes At mean 21 mo
Douglas [69]	131	0.1–7.1	Established persistent Pa	ELISA	<3.1 AU: −ve >3.1 AU: +ve	St-Ag:1–17	91[a] 53[b] 82[a]	64[a] 82[b] 57[a]	14[a] 26[b] 11[a] 19[b]	99[a] 94[b] 98[a] 98[b]	Baseline and exacerbations	No
					1:256[e]	ExoA in house	93[b]	52[b]				
Pressler [74]	791	0.3–73.2	3 grps: Free of Pa, Intermittent, and chronic Pa	CIE	>2 precipitins: +ve	St-Ag:1–17	96	89	87	97	Baseline and 1 year	Yes Seropositivity demonstrated risk factor for developing chronic Pa ($p < 0.05$)
				ELISA	>110 EU < 4 yrs. >160 EU for >4 yrs. 1.501 EU = value of 1	ExoA	93	89	86	95		
						St-Ag: 1–17	97	83	80	98		
Ratjen [41]	375	1.0–52.0	3 grps: free of Pa, intermittent, and chronic Pa	ELISA	1000 285 300	ExoA AP Ela 3 combined	72.0 85.4 76.2 92.7	97.5 97.5 97.5 93.3	95.9 96.6 96.2 92.1	80.8 88.8 83.2 93.8	Grp 1: baseline and after intervention therapy over last 8 yrs. Grp 2: patients who failed to clear Pa	Decrease in titres against AP and ExoA in patients clearing Pa infection, increasing in patients failing to eradicate Pa
Kappler [88]	183	0.4–41	3 grps: free of Pa, intermittent and chronic Pa	ELISA	<1:500: −ve >1:500: +ve	3 combined	86.1	95.6	97.1	80.2	3 monthly samples over 2 yrs	Seropositivity strongly linked to Pa infection
Proesmans [101]	162	1.3–52.8	4 grps: never, free of Pa, intermittent, chronic	ELISA	17 AU for chronicity	St-Ag: 1–17	88.0	96.0	92.1	93.9	1 serum sample outside resp. exacerbation	Seropositivity sig high in chronic Pa
Tramper-Stranders [71]	220	0.0–65.0	3 grps: not colonised, intermittent, and chronic Pa	ELISA	1:500 borderline +ve 1:1250 + ve	ExoA AP Ela	79 76 87	89	44–83 (range)	85–97 (range)	At end of study period	Yes Seropositivity demonstrated risk factor for developing chronic Pa

(*continued on next page*)

Table 4 (*continued*)

First author year	Patients (n)	Age range (yrs)	Pa time sampling	Method	Titre cutoff	Antigen	% sens	% spec	PPV	NPV	Antibodies assessed during Rx and at follow-up?	Evidence of seropositivity/increase in titres
Burns [111]	42	2.5–15.5	'initial' isolation within study period	ELISA	NR	ExoA	78	NR	NR	NR	Baseline and 3-monthly until 3rd bronchoscopy	Combined culture and serology more effective in determining infection than culture alone
				Immunoblot	1 band	Whole-cell proteins	90	NR	NR	NR		
Ramsey [81]	43	0.4–25.0	2 grps: expectorators; non-expectorators	ELISA	>1:800	ExoA	75[A] 46[B]	80[A] 93[B]	90[A] 83[B]	57[A] 70[B]	During 2 yr study period	ExoA good predictor of Pa colonisation, not sig when included with OP culture in regression model
Pedersen [112]	243 CF 164 con-trols	0.9–38 0–64	Chronic Pa + ve monthly sputum for 6 consecutive mo	ELISA CIE	≥1.65 EU: +ve ≥2 precipitins: +ve	St-Ag:1–17 St-Ag:1–17	90 100	100 98	NR NR	NR NR	During study period	Sig increase in precipitating antibodies with duration of infection
Döring [92]	10	8.0–29.0	Chronic Pa + ve monthly sputum for 6 consecutive mo	CIE	≥2 precipitins: +ve	St-Ag:1–17	100	NR	NR	NR	Multiple samples over 10 yr period	Following antibody response: AP response 15 mo Ela response 11 mo St-Ag response 6 mo an increase in titres and precipitins was observed in all patients
				RIA	1:10 1:10	AP Ela	100 90					
Høiby [113]	133	>4 yrs. Range NR	Patients colonised with Pa Chronic Pa: n = 38	CIE	≥2 precipitins: +ve	St-Ag:1–17	93	NR	NR	NR	Assessed at monthly clinic visits	Number of precipitins increased sig in 1st year of colonisation ($p < 0.01$); high number of precipitins assoc. with poorer VC ($p < 0.002$)

AP: alkaline protease; AU: area under the ROC curve; CIE: Crossed immune electrophoresis; ELA: elastase, ELISA: Enzyme-linked immunosorbent assay; EU: ELISA unit; Exo/ExoA/S/T: exotoxin A/S/T; grp/s: group/s; IgG: immunoglobulin G, MAg: multiple antigenic blend; MC: monoclonal antibody; mo: month/s; n: number; NPV: negative predictive value; NR: not reported; OP: oropharyngeal; p: p-value; pa: *Pseudomonas aeruginosa*; PopB: PopB protein; PopD; PopD protein; PPV: positive predictive value; RIA: radioimmunoassay; ROC: receiver operator characteristic curves; sig: significant; St-Ag: standard antigen; yr/s: year/s; VC: vital capacity; −ve: negative; +ve: positive; A: expectorators; B: non-expectorators.

[a] Patients undergoing BAL.

[b] Patients of the Australasian BAL Trial.

[c] Antigens produced by the Medical College of Wisconsin (MCW): ExoS + PopB, Cell lysate PAo1 and ExoA.

[d] Antigen commercialized by Statens Serum Institute (Copenhagen, Denmark).

[e] 1:256 dilution of serum.

Table 5
Anti-Pa antibodies at initial isolation of Pa infection.

First author year	Patients (n)	Age range (yrs)	Pa time sampling	Method	Titre cutoff	Antigen	% sens	% spec	PPV	NPV	Antibodies assessed during Rx and at follow-up?	Evidence of seropositivity/increase in titres
Daines [12]	261	0.0–12.0	1st lifetime	ELISA	<100: −ve >100: +ve	ExoA AP Ela 3/3	54.4 35.3 32.4 14.7	75.9 75.0 84.2 95.6	32.7 23.3 30.6 41.7	88.6 84.3 85.3 83.9	Baseline and follow up (annual samples over 3 yr period)	Yes at 6 mo No at 12 mo
Anstead [72]	303	1.0–12.0	1st lifetime	ELISA	<1:500: −ve >1:500: +ve	Pooled across 4 time-points AP3 Ela ExoA MC antigens* PopB +ExoS PAO1	 45 52 67 69 48 60	 81 78 58 65 83 72	 38 38 29 33 42 35	 85 86 87 89 86 88	4 time-points: wks 0,22,46 and 70	Seropositivity to AP and ExoA sig assoc. with increased risk of recurrent Pa during 60-week post eradication follow up (p = 0–003, p = 0.001)
Ratjen [41]	375	1.0–52.0	3 grps: free of Pa, intermittent and chronic Pa	ELISA	1000 285 300	ExoA AP Ela 3 combined	72.0 85.4 76.2 92.7	97.5 97.5 97.5 93.3	95.9 96.6 96.2 92.1	80.8 88.8 83.2 93.8	Grp 1: baseline and after intervention therapy over last 8 yrs. Grp 2: patients who failed to clear Pa	Decrease in titres against AP and ExoA in patients clearing Pa infection, increasing in patients failing to eradicate Pa
Kappler [88]	183	0.4–41	3 grps: free of Pa, intermittent and chronic Pa	ELISA	<1:500: −ve >1:500: +ve	3 combined	86.1	95.6	97.1	80.2	3 monthly samples over 2 yrs	Seropositivity strongly linked to Pa colonisation
Burns [111]	42	2.5–15.5	'initial' isolation within study period	ELISA Immunoblot1	>1:800	ExoA Whole-cell proteins	30 50	NR	NR	NR	Baseline and 3-monthly until 3rd bronchoscopy	Combined culture and serology more effective in determining infection than culture alone

AP: alkaline protease; AUROC: area under the ROC curve; CIE: Crossed immune electrophoresis; Ela: elastase, ELISA: Enzyme-linked immunosorbent assay; EU: ELISA unit; Exo/ExoA/S: exotoxin A/S; grp: group; IgG: immunoglobulin G, MAg: multiple antigenic blend; MC: monoclonal antibody; mo: month/s; n: number; NPV: negative predictive value; NR: not reported; Pa: *Pseudomonas aeruginosa*; PopB: PopB protein; PopD; PopD protein; PPV: positive predictive value; RIA: radioimmunoassay; ROC: receiver operator characteristic curves; sens: sensitivity; Spec: specificity; St-Ag: standard antigen; yrs.: years; −ve: negative; +ve: positive.

sis of infection is based on respiratory sample analysis, usually performed on expectorated or induced sputum or on OP swabs [62–64,76] Therefore, sampling methods strongly influence the sensitivity of infection diagnosis. Any definition of first lifetime/early/new as well as chronic Pa infection should clearly describe sampling frequency. Sample types, collection frequency and correct identification of the pathogen can be standardised by the application of ECFS-CTN SOPs which must be adopted by all CTN centres.

A minimum standard of four respiratory samples per annum must be applied in order to produce a homogenous study population. This is of the utmost importance as chronic infection may be mistakenly labelled as 'intermittent' as currently defined by Leeds Criteria [77]. Indeed, it has been reported that over half of patients categorised as 'intermittent' using Leeds criteria may actually have underlying chronic Pa infection [77]. Moreover, mucoidy is an important characteristic to consider in the definition of chronic infection as Heltshe [60] demonstrated that the majority of children (87%) developed mucoid infection before meeting the 'Leeds' definition of chronic infection.

The sensitivity and the positive predictive value of OP cultures in detecting Pa infection is low when compared to BAL [79–81]. This limited sensitivity might result in missed detection of initial Pa infection. To increase sensitivity of detection in non-expectorating paediatric patients with CF, induced sputum samples have recently been proved to provide additional microbiological information in comparison to OP swabs [82] and very importantly BAL samples do not seem to be more sensitive than induced sputum in this population [66,83]. There is therefore no clear evidence to support the *routine* use of BAL for the diagnosis and management of pulmonary infection in children with CF [76,84]. Moreover, Pa qPCR might improve sensitivity of infection diagnosis on culture negative OP swabs [61,85,86]. However, this will require further comparative validation studies using BAL samples [87].

4.2. Anti-Pa antibodies

Anti-Pa antibodies have demonstrated success as a useful adjunct for assessment of early eradication therapy [72] and to distinguish intermittent from chronic Pa [88,89]. Daines [12] was the first to report the utility of Pa serology in predicting subsequent first isolation from cultures of Pa negative patients [12], and Hayes [68], Ratjen [41] and Pressler [74] have all reported high diagnostic accuracy of serology [41,68,74]. The presence of raised titres of anti-Pa antibodies (anti-alkaline protease and anti-Exotoxin A) before AET is associated with an increased risk of Pa recurrence [72]. Therefore, studies excluding participants with raised anti-Pa antibodies might be expected to achieve higher eradication rates and furthermore prevent enrolment of ineligible patients [90].

The use of serology raises many problems regarding the interpretation and the validity of anti-Pa antibodies, both in the early stages of infection and in monitoring AET. There is a need for a standardised method of Pa antibody testing. This would either require the same kit/method to be used in several labs or testing performed in a central laboratory with the same kit/method. The latter requires transport of blood samples, potentially over long distances.

The two kits mostly used in Europe for the detection of anti-Pa antibodies are the *Pseudomonas* -CF-IgG ELISA kit by Statens Serum Institut, Denmark, and the Mediagnost anti-*Pseudomonas aeruginosa* IgG Enzyme Immuno Assay E15, Reutlingen, Germany.

Since the antigens used (for example, ExoA, Ela, AP and St-Ag: 1–17) in different anti-Pa antibody detection kits are not consistent, their use may give variable results. In the absence of Pa infection, the optimum time interval for repeated checking of Pa antibody dynamics is not known or standardised. There is also inconsistency in the levels of anti-Pa antibody response detected by different kits as patients move between the different phases of Pa infection e.g. from time of initial Pa infection to chronic infection (Table 5).

A decrease in anti-Pa antibody titres after AET has been described. However, there may not be an immediate decline in antibody titres even after successful eradication because of the persistence of an antigen stimulus for a long period. This is linked to Pa antigens in obstructed airways despite negative Pa cultures. This makes anti-Pa antibody titres in the context of eradication definition and "next infection" or "intermittent infection" difficult to interpret. Host response to initial phases of Pa infection is variable and the chronology of the immune response to Pa antigenic determinants is not completely clear. The immune response to the virulence factors produced by Pa (Exotoxin A, Elastase and alkaline protease) seems to occur over a longer period in comparison with St-Ag, which may usually be expressed earlier [91,92].

Longitudinal immunological studies would therefore need to be undertaken to assess the utility of anti-Pa antibodies in monitoring response to repeated eradication treatment.

Cut-off values in the initial phases of Pa infection need to be determined by receiver operating characteristic (ROC) curve analyses [71]. These have only been agreed with certainty for chronic Pa infection. All these considerations highlight that unless sampling and laboratory issues are properly addressed it will be difficult to apply definitions in a robust fashion. This points to the need to establish a central laboratory capable of providing standardised anti-Pa antibody testing for all trial centres or the provision of the same standardised method for testing across a network of laboratories. No specific training requirements are needed if a central reference laboratory is used for Pa antibody testing.

4.3. Considerations for proposing a definition regarding initial (first lifetime/early/new) Pa infection to be used in clinical trials

Traditional methods of Pa identification reliant upon microbiology present inherent difficulties. Decision regarding the use of serology in defining Pa microbiological status introduces a greater level of complexity in terms of patient enrolment and laboratory testing. Therefore, in the initial phase of Pa infection (Table 2), serology should be considered an adjunct to, rather than a replacement for cultures.

Thus, the proposed definitions to describe initial (first lifetime/early/new) Pa infection in CF patients for application in clinical trials are:

"First lifetime documented Pa in a respiratory culture with anti-Pa antibody titres below the cut-off for chronic infection" (level of evidence 1+, grade of recommendation A) OR, if the patient has previously grown Pa *"New Pa detection in a respiratory culture after at least four negative cultures regularly (quarterly) performed over one year, with anti-Pa antibody titres below the cut-off for chronic infection"* (level of evidence 2+, grade of recommendation C).

4.4. Considerations for proposing a definition of Pa eradication to be used in clinical trials

'Successful' eradication may be difficult to define as it requires 'negative' cultures over a certain period of time, but its appropriate length is unknown. The Artimino Consensus Definition (11), "repeated Pa negative respiratory samples (at least three) in the six months after completion of the eradication regimen, in conjunction with negative serum anti-Pa antibody titres", had a clear time frame and was not solely reliant on cultures [11]. It also clarified the frequency of sampling required. However, it included the requirement for standardised antibody testing and the need to collect blood samples as previously highlighted.

Other purely culture-based definitions, such as the UK CF Trust Definition (2004) "at least three consecutive negative respiratory cultures spread over a six-month period", can be simple, have clear time frames, defined frequency of sampling and the absence of problems associated with antibody testing [93]. However, these are solely dependent on culture results and therefore may miss some therapeutic failures, particularly in non-expectorating patients.

Despite both definitions of eradication mentioned above being based on an observation period of six months, the concept of "sustained eradication" has been introduced to indicate that particular category of patients who maintained Pa negative cultures for 12 months after AET [39]. Those "sustained eradicators" proved to have a reduced risk (74%) of developing chronic infection with less anti Pa antibiotic usage and a reduced risk (57%) of mucoid Pa phenotype during a 5-year observation period, compared with non-sustained eradicators. During the same period of time, no statistically significant differences were observed with respect to other clinical parameters (lung function decline and pulmonary exacerbation) [39].

For use in clinical trials, the definition of Pa eradication, must therefore include both culture and PA serology.

"Pa negative cultures for 12 months after treatment (having performed quarterly cultures) in conjunction with a titre of anti-Pa antibody below the cut-off for chronic infection one year after treatment." [39] (level of evidence 2+, grade of recommendation C).

Serological studies performed one year after treatment are able to predict AET success or the evolution towards chronicity (Table 4).

Future studies should evaluate anti-Pa antibody dynamics over time as well as the molecular characteristics of bacterial isolates in order to distinguish new infections from re-colonisation with strains, which have been suppressed but not eradicated. Molecular genotyping, such as that described in the study by Kalferstova et al. [78], should be conducted to examine all Pa isolates obtained after early eradication treatment, to evaluate the recurrence of the same strain and the possible evolution towards chronic infection [78]. Studies on phenotypic and genotypic characteristics of bacterial strains responsible for initial infection and associated with treatment failure should be conducted. The molecular mechanisms of bacterial adaptation to the CF airways need more in-depth studies and the role of the paranasal sinuses in the development of chronic Pa infection should also be clarified. Paranasal sinuses are considered a reservoir for lung infection, therefore longitudinal studies aimed at clarifying the clinical relevance of paranasal sinuses' Pa microbiological status in determining chronic lung infection are necessary. Clinical trials to assess the efficacy of paranasal sinuses' medical/surgical treatments on the evolution towards chronicity also need to be performed [94–96].

Moreover, the role of lung damage in predisposing the patients to bacterial infection and the effect of modulators in reducing airways' receptivity to Pa infection require better assessment.

Future studies should also focus on diagnostic accuracy of induced sputum in comparison with other sampling methods in non-expectorating patients. Application of non-routinely used molecular techniques, such as qPCR, must also be incentivized, as this might help to discriminate between intermittent and chronic Pa infections [61,85,86].

4.5. Considerations for proposing a definition of chronic infection to be used in clinical trials

Times to chronic Pa infection and to first appearance of mucoid Pa phenotype infection have been considered as clinically relevant indicators [30,39].

In this case, the follow-up period would be years rather than months. Historical data from Copenhagen suggests that AET can delay progression to chronic infection by at least 3.5 years [23,97].

There are a number of published definitions of chronic Pa infection. For example, the Copenhagen definition requires the isolation of Pa from sputum or respiratory samples on two or more occasions extending over six months or a shorter period if accompanied by a substantial rise in anti-Pa antibodies [96]. However, it requires a frequency of respiratory sampling (e.g. monthly) which many CTN centres do not currently perform. The requirement for antibody testing has previously been discussed. It would also require a clear definition of what is meant by a 'substantial rise' [98].

As another example, the Leeds Criteria definition does not require systematic sampling as practiced in Copenhagen or access to blood samples and antibody testing. However, the frequency of sampling required is not made clear and it may miss some chronically-infected patients, particularly if the majority of samples collected are OP swabs. Therefore, some chronically Pa infected patients are erroneously categorised by Leeds Criteria as 'Intermittent'. The Leeds Criteria also make no allowance for raised anti-Pa antibodies or the appearance of the mucoid phenotype of Pa, both of which may indicate the transition from early colonisation to chronic infection. At present the UK and the Cystic Fibrosis Foundation Registries accept the Leeds Criteria definition [77,93]. As there is a good correlation between chronic *Pa* infection and anti Pa-antibodies, the ECFS Registry accepts both the Leeds Criteria definition as well as definitions based on cultures associated with significantly raised anti-Pa antibodies according to local laboratory results [98].

Regarding chronic infection, the accepted definition appears to be the Leeds definition [77] (level of evidence 2++, grade of recommendation B). When the Leeds criteria for chronic infection are ambiguous, for instance, due to an insufficient number of samples to assess the immediate Pa status or when a patient has a questionable long-term history of intermittent Pa status, only patients with repeated culture positivity for Pa and a titre of anti-Pa antibodies above the cut-off for chronic infection should be included in the clinical trials.

It is often difficult to distinguish the intermittent from the chronic phase. Therefore, as stated above, future studies should aim to evaluate phenotypic and genotypic characteristics of bacterial strains during their transition to chronic status [99]. New methods (qPCR) could be useful in discriminating between intermittent and chronic Pa infection [61,85,86]. Recently, Jonckheere et al. [100] reported that genotyping was more sensitive than European Consensus Criteria in establishing the diagnosis of chronic infection [100].

4.6. Advocacy for Pa serology

Anti-Pa serology relating to first lifetime/early/new Pa infection and successful Pa eradication introduces a greater level of complexity in terms of patient enrolment, laboratory testing and outcome measure. Currently, the optimum method of antibody testing, and the interpretation of the antibody titres in the initial phase of infection are not clear. Specific research into these topics should be conducted.

The follow-up of patients who had undergone AET may require repeated blood sampling to observe antibodies' dynamics and to ensure their correct interpretation. Therefore, an agreed approach regarding frequency of blood sampling and interpretation of results has to be established. On balance, the evidence supports the use of anti-Pa antibodies as a biomarker of progression to chronic infection. Serology performed one year after treatment could provide useful indications on the outcome and thus complement culture results in an attempt to successfully monitor Pa recurrence. Resolution of the debate surrounding Pa serology will require longitudinal studies with improved serological methods and more frequent sampling. It would also be helpful if there were further studies comparing the relative performance of different commercial anti-Pa antibody kits to inform the adoption of the most appropriate one for use in CF.

Regarding chronic Pa infection, until now no definitions have been subject to head-to-head comparison. 'Leeds Criteria' have been clinically validated [77,101], but they have not been compared to other definitions, particularly those that include anti-Pa antibodies. As some Pa chronically-infected patients are erroneously categorised by Leeds criteria as "intermittent", the accuracy and precision of such categories could be enhanced by the results of anti-Pa antibody testing, mainly in non-expectorating children.

4.7. Definitions in the context of evolving technology

Very importantly, the definition of 'Pa positive' or 'Pa negative' respiratory specimens relies on the sensitivity threshold to recover *P. aeruginosa*. Next Generation Sequencing (and qPCR) may change the threshold of detection as compared to standard culture-based microbiology in the near future [102,103]. Such technological advances will have a major impact on our understanding of CF microbiology. However, at present, this requires in-depth knowledge in genomics and big data and is not readily available in all CF centers. Therefore, microbiological culture is still the gold standard for defining whether a patient is classed as Pa 'positive' or 'negative'. If metagenomics or PCR become routinely used in microbiology diagnostic laboratories, then the definitions will be reviewed and revised accordingly.

5. Conclusion

Having an accepted definition of successful eradication is crucial in determining who is eligible for enrolment in future AET trials. Unless we enrol subjects, who have never previously grown Pa, the inclusion criteria for AET trials have to be consistent and complementary.

Microbiological monitoring through regular sampling of suitable respiratory samples and standardization of culture methods remain essential requisites on which to set up microbiological definitions.

All ECFS-CTN centres enrolling patients will need to follow the same standards for frequency and method of sample collection and isolation and identification of Pa during routine clinical practice. Serology should be incorporated in the definitions of initial infection and eradication used in clinical trials to better classify patients at enrolment mainly in non-expectorating children. The Leeds definition is appropriate for chronic Pa infection.

The proposed definitions, aimed at improving clinical and laboratory practices, are crucial in order to better classify patients at enrolment in clinical trials and to harmonize patients' follow-up throughout Europe. In summary, a proposed list of critical gaps in knowledge and/or research priorities:

5.1. Critical gaps in knowledge and/or research priorities

- Evaluation of diagnostic accuracy of different sampling methods.

- Improvement of the sensitivity of *P.aeruginosa* infection diagnosis methods by Next Generation Sequencing and/or qPCR to detect first *P.aeruginosa* infection or to discriminate between intermittent and chronic *P.aeruginosa* infections.
- Evaluation of phenotypic and genotypic characteristics of *P.aeruginosa* strains associated with initial infection, treatment failure or transition to chronic status.
- Genotyping of all *P.aeruginosa* isolates in order to distinguish new infections from re-colonisation with strains suppressed but not eradicated.
- Evaluation of anti-*P.aeruginosa* antibody dynamics over time, in order to monitor the response to early eradication treatment and to predict evolution towards chronicity.
- Evaluation of performance of different commercial anti-*P.aeruginosa* antibody kits.
- Role of the paranasal sinuses in the development of chronic *P.aeruginosa* infection.
- Role of lung damage in predisposing the patients to bacterial infection.
- Effect of modulators in reducing airways' receptivity to *P.aeruginosa* infection.

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Appendix: members of the ECFS-CTNMicrobiology Group

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