

SAMPLE MANUSCRIPT

Comparative diffusion assay to assess efficacy of topical antimicrobial agents against *Pseudomonas aeruginosa* in burns care

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Comparison of diffusion assays to assess the efficacy of topical agents against *Pseudomonas aeruginosa* in the care of burn patients

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Susceptibility of a resident *Pseudomonas aeruginosa* clone to different topical antimicrobial agents according to different diffusion assays

English

Scientific style

Methods and reporting

15

Writing / Organization / Scientific validity

ABSTRACT

Background

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Severely burned patients may develop life-threatening nosocomial infections due to *Pseudomonas aeruginosa*, / *Pseudomonas aeruginosa*, which can exhibit a high-level of resistance to antimicrobial drugs and has a propensity to cause nosocomial outbreaks. Antiseptic and topical antimicrobial compounds constitute / are major resources for burns care but in vitro testing of their activity is not performed / done in practice.

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Results

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In our burn unit, a *P. aeruginosa* clone multiresistant to antibiotics colonized or infected 26 patients over a 2-year period. This resident clone was characterized by PCR based on ERIC sequences. We investigated the susceptibility of the resident clone to silver sulphadiazine and to the main topical antimicrobial agents currently used in the burn unit. We proposed / developed an optimized diffusion assay used for comparative analysis of *P. aeruginosa* strains. The resident clone displayed lower susceptibility to silver sulphadiazine and cerium silver sulphadiazine than strains unrelated to the resident clone in the unit or unrelated to the burn unit.

Conclusions

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The diffusion assay developed herein / we developed detects differences in behaviour against antimicrobials between tested strains and a reference population. The method could be proposed for use / may be useful in semi-routine practice of / in medical microbiology.

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Keywords: *Pseudomonas aeruginosa*, burns, silver sulphadiazine, antiseptics, ERIC-PCR, diffusion assay

BACKGROUND

The current techniques of resuscitation, surgery and wound care have significantly improved / reduced the morbidity and the mortality of patients with burn wounds [1].

5 However, severely burned patients may still develop life-threatening nosocomial infections that remain a major challenge for burn teams [2]. The most frequent infections are wound infections, pneumonia, bloodstream and urinary tract infections [2,3]. Among the nosocomial pathogens, *Pseudomonas aeruginosa* from the patient's endogenous microflora and/or from the environment represents the most common /
10 commonly isolated bacteria in many centres [2,4,5]. Infections with *P. aeruginosa* are particularly problematic since this bacterium exhibits inherent tolerance to several antimicrobial agents and can acquire additional resistance mechanisms turning inefficient all current antimicrobial drugs / mechanisms, making all current antimicrobial drugs inefficient [6,7].

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Antiseptic and topical antimicrobial compounds represent major resources in the therapeutic arsenal available for burns / burn care. It is widely recognized that these agents have played a significant role in decreasing the overall fatality rate in burn units. Some of them such as povidone-iodine and chlorhexidine are used for antiseptics
20 during wound care, therapeutic bathes, debridement and surgery. Others, prepared as ointment / as an ointment or unguent, provide antimicrobial effects associated to the 'mechanic' / 'mechanical' protection of the wound. For example, the use of cerium nitrate-silver sulphadiazine that / sulphadiazine, which forms a leather-like eschar on burn wounds, allows surgical treatment to be delayed and enables sequential excision
25 and grafting [8,9,10]. This wound treatment policy is supposed / assumed to improve the patient / patient / (No adjective needed since 'survival' is understood to refer to the patient.) survival [8,11] and is increasingly used. / used with increasing frequency.

Resistance / The resistance of *P. aeruginosa* to silver sulphadiazine has been
30 previously documented [12]. In our unit, a *P. aeruginosa* clone multiresistant to antibiotics colonized or infected 26 patients over a 2-year period. Silver sulphadiazine susceptibility of this clone / The susceptibility of this clone to silver sulphadiazine was questioned owing to long-time / prolonged colonization or to / (*) refractory infections of the wounds. / wound infection. We comparatively investigated / compared the
35 susceptibility of the resident clone and unrelated *P. aeruginosa* strains to silver sulphadiazine and to the main topical antimicrobial agents currently used in the burn unit. For this purpose, we developed an optimized rapid method based on diffusion

assay. / a diffusion assay. This method appears suitable for semi-routine / the semi-routine investigation of therapeutic failure or outbreak situation / situations // therapeutic failures or outbreaks in burn unit / the burn unit and may be used to guide the choice of the most appropriate topical antimicrobial agent for patient's management. / for burn patients.

MATERIAL AND METHODS

Patients, settings, samples and bacterial strains

The burn unit of the Academic Hospital of Montpellier is a French regional centre. The ward displays / comprises 6 intensive care unit rooms, 4 hospitalization rooms and 2 bathrooms. For microbiological analyses, serial samples are taken on admission to the intensive care unit or whenever required for clinical reasons. Extensive environmental samplings including water and surfaces are performed twice a year or whenever required during epidemic alerts. We retrospectively analysed strains of *P. aeruginosa* isolated from patients admitted to the burn unit from January 2005 to August 2007 as well as strains recovered from environment / the environment during the same / study period. All the / (*) culturable strains (n=87) were included in the study. Thirteen strains of *P. aeruginosa* unrelated to the burn unit obtained from a collection of clinical strains were also included.

Routine antimicrobial treatment of patients in the burn unit

Silver sulfadiazine (SSD), Flammazine® (1% SSD) or Flammacerium® (1% SSD + 2.2% cerium nitrate), / (no comma between subject and predicate) is generally applied each two days. Mafenide acetate (Sulfamylon®) (For specific brand names or commercial names, the name of the manufacturer, city and country must be identified.) is occasionally used. Povidone iodine is used for wound rinsing during dressing and surgery. Patients are bathed every two days with water containing chlorhexidine. If a *P. aeruginosa* infection is suspected, the first-line treatment is piperacillin / // or (the meaning of / is not clear) tazobactam plus tobramycin.

Microbiological analysis

The bacteria were isolated from clinical or environmental samples by standard microbiological procedures. *P. aeruginosa* / *Pseudomonas aeruginosa* was identified using / with Gram staining, positive oxidase reaction, production of pigments onto / on King A and King B media (Bio-rad Laboratories) or API 20NE system (bioMérieux). // with positive Gram staining, a positive oxidase reaction, pigment production in King A or King B media (Bio-rad Laboratories) or ___ ?? ___ in the API 20NE system

(bioMérieux). The bacterial strains were stored at -80°C / -80°C in a preservative medium (bacterial preservers, / Bacterial Preservers, Technical Service Consultant Limited).

5 Pulsed-field gel electrophoresis (PFGE) and ERIC-PCR typing

Pulsed-field gel electrophoresis (PFGE) after digestion by *SpeI* was performed as previously described [13]. The ERIC-PCR assay was performed as described by Mercier (1996) [14] with modifications. DNAs were extracted using the kit AquaPure Genomic DNA kit (Bio-rad Laboratories) as recommended by the supplier.

10 Enterobacterial repetitive intergenic consensus (ERIC) PCR conditions were validated using unrelated, closely related and identical isolates of *P. aeruginosa* (as determined by PFGE). ERIC-PCR was performed using 0.5 ml / ml thinwalled / thin-walled PCR tubes in an Eppendorf MasterCycler® thermal cycler. The reaction mix contained the following reagents: 2.5 U of / (*) GoTaq Flexi DNA polymerase (Promega) in / in an
15 appropriate buffer with 2 mM MgCl_2 and 3.5% DMSO, 0.2 mM each deoxynucleoside triphosphate (Fermentas), 20 pmol of each primer (ERIC1 5'-CACTTAGGGGTCCTCGAATGTA-3', ERIC2 5'-AAGTAAGTGAAGTGGGGTGAGCG-3') and 50 ng of genomic DNA. The final reaction volume was adjusted to 50 μL . PCR amplification was performed with an initial denaturation step at 95°C / 95°C for 3 min
20 followed by 30 cycles of denaturation (90°C for 30 s), primers / primer annealing (45°C for 1 min) and extension at 72°C for 4 min with a final extension at 72°C for 16 min. Amplicon (5 μL / 5 μL) was loaded with 6X / 6x loading buffer (50% saccharose, 0.1% bromophenol blue) into a 1.5% agarose gel in 0.5X / 0.5x Tris-Borate-EDTA (TBE)
(abbreviation not needed because the term appears only once in the manuscript)
25 buffer with 0.5 $\mu\text{g mL}^{-1}$ ethidium bromide. Electrophoresis was run at 80V / 80 V for 3h / 3 h at room temperature. PFGE profiles were visually interpreted as follows: when two profiles were identical or differed by 3 or less / fewer than 3 DNA fragments, the same letter was affected to the profiles. / the profiles were identified by the same letter. PFGE profiles differing by more than 3 bands were identified by different letters. The
30 same nomenclature was used for ERIC profiles but numbers were used instead of letters.

Antimicrobial susceptibility testing

Antibiotic susceptibility was tested by disk diffusion assay on Mueller-Hinton agar and
35 interpreted according to the recommendations of the Antibiogram Committee of the French Microbiology Society (CA-SFM) (http://www.sfm-microbiologie.org/UserFiles/file/CASFM/casfm_2010.pdf). The antibiotics / antibiotic

disks used (BioRad, Marne-la-Coquette, France) (**The city and country should be given for all materials and apparatus.**) were as follows: ticarcillin (75µg), ticarcillin / clavulanic acid (75 µg /10 µg), piperacillin (75µg), piperacillin / tazobactam (75µg /10µg), imipenem (10µg), cefotaxime (30 µg), ceftazidime (30µg), cefepime (30µg),
5 aztreonam (30µg), gentamicin (10 UI / IU), tobramycin (10µg), nalidixic acid (30µg), ciprofloxacin (5µg), fosfomycine / phosphomycine (50µg). Colistin Minimal Inhibitory Concentration (MIC) was determined using Etest® (AB BIODISK, Solna, Sweden) according / according to the CA-SFM protocol. Identification of resistance mechanisms was deduced from susceptibility testing by disk diffusion assay results according to
10 Courvalin et al. [15]. Susceptibility to topical antimicrobial agents was tested by / by the agar well diffusion (AWD) assay modified from Nathan et al. [16]. The surface of 5-mm-thick Mueller-Hinton agar plates was inoculated with a bacterial suspension visually adjusted to 0.5 McFarland (108 CFU/mL) and diluted 100 fold / 100-fold. Then, 8-mm diameter / 8-mm-diameter holes were made in agar plates with / with a sterile die cutter
15 and the wells were loaded with topical agents. (new paragraph needed here)

The following topical agents were tested: 1% SSD (Flammazine®, Solvay), 1% SSD + cerium nitrate (SSDC) (Flammacerium®, Solvay), 5% mafenide acetate (Sulfamylon®),
20 10% povidone-iodine (Betadine Gel®), 10% povidone-iodine (alcoholic solution) and chlorhexidine. Before loading, Betadine gel, SSD and SSDC were diluted at 1/2, 1/4 and 1/4 w/v / w/v, respectively, in sterile distilled water to insure the reproducibility of pipetting. Aliquots of the commercialized products were weighted in microtubes in sterile conditions, conserved as recommended by the supplier and diluted
extemporaneously. Then, wells were loaded with 150 µl / µL of the diluted agent. This
25 volume insured complete well loading with homogeneous contact between the agent and the well edge. The inhibition diameters were measured after 18h of incubation at 37°C using the / an Antibiotic Zone Reader apparatus (Fisher Lilly).

Statistical analysis

30 Analyses were performed either in duplicate or in triplicate in independent assays. (Which analyses were done in duplicate and triplicate should be specified.) For each strain and each antimicrobial agent, the mean inhibition diameter and the / (*) standard deviation were calculated. Differences in inhibition zone sizes between groups of strains were tested using Student's t-test. P<0.05 was taken as statistically
35 significant.

RESULTS

Microbiology and / (*) antibiotics / Antibiotic resistance of the P. aeruginosa isolates

A total of 100 P. aeruginosa isolates, including 67 clinical and 33 environmental isolates / isolates, were available for retrospective analysis. Eighty-seven isolates were recovered from 26 hospitalized patients (n=55) or from environment / the environment (n=32) in the burn unit. Thirteen additional isolates corresponding to 12 clinical samples and to / (*) 1 environmental sample formed a / the collection of hospital isolates epidemiologically unrelated to those of the burn unit. Origin / The origin of the isolates was / is given / shown in Tables 1, 2 and 3.

Forty-two isolates of / from the burns / burn unit displayed antimicrobial susceptibility profiles with resistance to about / almost ?? all commercially available antibiotics tested. Among them, eighteen / 18 clinical and 3 environmental strains resisted / were resistant to all beta-lactams including imipenem, to aminoglycosides, to ciprofloxacin and to fosfomycin. This multi-drug resistance pattern will be / is named MDR1 hereafter (Table 1). Closely / A closely related pattern, named MDR2, grouped 10 clinical and 11 environmental strains resistant (R) to all antibiotics tested but susceptible (S) to fosfomycin (Table 1). For the / (*) strains with MDR1 / the MDR1 and MDR2 phenotype, the colistin MIC value was from 4 to 8 µg/mL. No MDR1 or MDR2 phenotype was observed in the unrelated strains collection. / None of the unrelated strains had the MDR1 or MDR2 phenotype. Other isolates from the burns / burn unit or not / and elsewhere (Tables 2 and 3) showed various resistance patterns. Regarding beta-lactams, we observed wild type / the wild-type phenotype, cephalosporinase overexpression, penicillinase production, oxacillinase production, efflux pumps / pump overexpression, porin D2 impermeability or complex phenotypes associating several of the previous resistance mechanisms. The strains displayed various behaviours against fluoroquinolones, aminoglycosides and fosfomycin. (Should these "various" behaviours be explained in more detail, or summarized in a Table? Were these behaviours unexpected in any way or perhaps inconsistent with the other resistance/susceptibility patterns reported earlier in this paragraph?)

Molecular typing of P. aeruginosa

We analysed all the / the entire bacterial population (n=100) by ERIC-PCR and a comparison to PFGE was performed for / these results were compared with the PFGE findings for about one third of strains / the strains (n=33). Interpretable / An interpretable ERIC-PCR pattern was obtained for all isolates. A gel representative of the ERIC-PCR patterns is shown in Figure 1. (new paragraph needed here)

The strains were distributed in 36 distinct ERIC-PCR profiles (Tables 1, 2 and 3). PFGE confirmed the ERIC-PCR-based clustering (Table 1 and 2) for the 33 strains analysed by both methods, thereby validating the PCR-based results. The 55 clinical strains and **the / (*)** 32 environmental strains displayed 17 and 11 profiles different ERIC-PCR profiles, **respectively.**// **The 55 clinical strains displayed 17 different ERIC-PCR profiles, and the 32 environmental strains displayed 11 profiles.** The strains unrelated to the burn unit were more diverse since 12 different profiles were observed for the 13 strains. A main ERIC-PCR profile type, named ERIC1, was observed for 42 isolates corresponding to 28 clinical strains isolated from 13 different patients and 14 environmental isolates from the **burns / burn** unit (Table 1). The ERIC1 profile was never found in strains unrelated to the burn unit. **The / (*)** **Strains** with **ERIC1 / the ERIC1** profile **have been / were** isolated **from / between** February 2005 **to / and** April 2007. All these isolates were multi-resistant to antibiotics and displayed the **resistance pattern MDR1 or MDR2. / MDR1 or MDR2 resistance pattern.** The 45 other isolates from the **burns / burn** unit displayed 23 other different ERIC-PCR patterns, and none of them **were of / had the** MDR1 or MDR2 phenotype (Table 2 and 3). **Out / Among** **isolates** of the ERIC1-type group, the strains sharing the same **ERICPCR / ERIC-PCR** profile were isolated from the same burn patient, and the same ERIC-PCR profiles were not shared between clinical and environmental strains in the burn unit. The strains unrelated to the burn unit displayed ERIC-PCR patterns that were not observed in the burn unit. **Again, / (*)** **In this** group, the same pattern was obtained only for strains isolated from the same **patients. (Should this be "patient"?)** **Finally, / (*)** **Genomotyping / Genome typing** showed that MDR1 and MDR2-type strains **are / were** clonal and that this clone persisted over a **2-years / year** period in the burn unit.

Optimization of the agar well diffusion (AWD) / (*) assay for topical agents

The wells were filled with agents in their commercial forms except for semi-solid forms, which **need / needed** to be diluted to insure **the / (*)** reproducibility of the **wells / well** pouring. A range of binary dilutions from pure to 1/8 was tested on 5 selected bacterial strains. The resulting inhibition diameters did not vary significantly for Flammazine® (from 17 to 15 mm) **and / or** for Flammacerium® (from 20 to 18 mm). For Betadine® gel, the range of inhibition **zone / zones** was **wider, / larger**, from 27 to 20 mm **when the dilution increase. (meaning unclear)** The absence of defined cut-off values for inhibition diameter in AWD assays imposed a comparative approach **for the results interpretation. / to interpret the results.** **Therefore, attention should be given to the reproducibility of the method rather than to the absolute diameter measuring. (This**

belongs in the Discussion section.) In all cases, the edges of the inhibition zones were more regular and clear / clearer when the agents were diluted. We chose for each agent / For each agent we chose the lowest dilution insuring / that ensured (“Insuring” could be misunderstood to mean “and we thereby ensured”. It is not clear

5 whether the subject of “insuring” is “we” or “the lowest dilution”.) easy and reproducible pipetting and wells / well pouring: 1/2, 1/4 and 1/4 w/v for Betadine gel®, SSD and SSDC respectively. / 1/2 w/v for Betadine gel®, 1/4 w/v for SSD and 1/4 w/v for SSDC.

10 The AWD method has also been / was improved by testing different bacterial inoculums. Bacterial charge / load affected significantly / significantly affected the diameter of inhibition (data not shown). This was particularly obvious for the Sulfamylon® diameter, which was large (>40 mm) and not clearly delimited, with micro-colonies growing in / at / along the border of the main diameter. Inoculation of the
 15 plates with 106 CFU gave the more / most easily interpretable results. With this inoculum, clear-cut and easy to read / easy-to-read diameters were obtained for all topical agents. Particular care should be taken for the preparation of the inoculum in order to insure reproducibility of the AWD tests. This optimized protocol is compatible with a semi-routine practice of medical microbiology since about 10 strains could be
 20 analysed over a 1-hour period of bench manipulation, including dilution of commercialized agents aliquots. (**This belongs in the Discussion section.**)

Activity of the topical antimicrobial compounds

Since the method AWD / AWD method was not standardized and reference strains
 25 were unavailable for antimicrobial assays on / of topical agent, we undertook AWD assays with comparison of results at the population level. / we compared the results of our AWD assays at the population level with those from another study. First, the mean inhibition diameter for each topical agent was compared with the results of Pirnay et al. [12] (comma removed) at the whole population level. Mean diameter for SSD, SSDC,
 30 chlorhexidine, iodine-povidone and Sulfamylon® were respectively 19.7 mm, 19.4 mm, 19.3 mm and 44.9 mm in our study and 20.2 mm, 21 mm, 19.1 mm and >30 mm in the study of Pirnay et al. [12]. (A simple table is better)

Study	SSD	SSDC	Chlorhexidine	Iodine-povidone	Sulfamylon®
Present study	19.7 mm	19.4 mm	19.3 mm	44.9 mm	??
Pirnay et al. [12]	20.2 mm	21 mm	19.1 mm	>30 mm	??

(Making the table revealed that the data are missing for one of the agents, but which agent is missing cannot be determined from the text.)

5 The similarity of the mean diameters in two **population / populations** of *P. aeruginosa* isolated in **burns / burn** units **gave arguments to validate / supported the validity of** our AWD approach.

Secondly, **undertook a comparative AWD assay / we compared our AWD results** between isolates belonging to the MDR1/2- ERIC1 clone (group 1; n=42) and unrelated
10 *P. aeruginosa* strains from the **burns / burn** unit (group 2; n=45) or from elsewhere (group 3; n=13). The results of the comparative AWD tests **were / are** presented in **T**ables 1, 2 and 3 and summarized in Figure 2. **(new paragraph here)**

The isolates belonging to group 1 displayed **significant decrease of / significantly**
15 **smaller** SSD and SSDC inhibition diameters **comparatively / compared to group / groups** 2 and 3 ($P < 0.001$) (Figure 2). For chlorhexidine, iodine-povidone and Sulfamylon® no significant differences in inhibition diameters were observed among the 3 groups ($P > 0.05$) (Figure 2). In spite of **a / the** selective pressure **of / exerted by** topical agents **similar to / as in** group 1, **(meaning unclear)** most of the group 2 strains
20 displayed inhibition diameters corresponding to those observed in **the / (*)** group 3 for all agents tested. However, 4 strains affiliated to group 2 (PAB27, PAB38, PAB41, PAB55) showed inhibition diameters similar to **strains / the strains of / in** group 1. **The / (*)** Strains PAB38 and PAB41 isolated from the same patient displayed the ERIC-PCR 11 profile and a **wild type / wild-type** phenotype regarding **the / their** resistance to
25 antibiotics. This indicated that the low susceptibility to SSD and SSDC was not **obligatory / necessarily** associated with multi-resistance to other antimicrobial agents. **The / (*)** Isolate PAB55, belonging to **the / (*)** ERIC-PCR profile 10, also showed **limited / a smaller** diameter around SSD and SSDC wells and a **wild / wild-type** phenotype regarding **antibiotics. / antibiotic resistance**. In the same ERIC group, **the / (*)** strain
30 PAB49 was isolated from the same patient **one month before. (before when?)** This isolate did not display reduced susceptibility to topical agents but displayed **a / the** phenotype of **penicillinase / a penicillinase** producer. **(new paragraph here)**

Other / Another situation **was illustrated by the / (*)** strains PAB27 and PAB28, which
35 **shared the genototype ERIC6 / ERIC6 genome type and** were isolated on the same day from burn wounds of **the patient 15. / the same patient. The 2 / Both** strains presented the same **wild / wild-type** antibiotypes, but PAB27 **only showed / showed**

only limited diameter / inhibition diameters around SSD and SSDC. This suggested that in a same genomotype the resistance patterns to antibiotics and/or topical antimicrobial agent could vary rapidly. Another hypothesis was the co-existence of mixed populations harbouring diverse phenotypes against antimicrobial agents. (This belongs in the Discussion.)

DISCUSSION

We proved / showed by PFGE and ERIC-PCR that 42 strains isolated from the environment and from the / (*) patients of the / a burn unit over a 2-year period belonged to the same clone. They / All strains displayed the multi-drug resistant phenotypes MDR1/2. / MDR1 or MDR2 phenotype. Comparison of PFGE to recent sequence-based typing methods such as Multi-Locus Sequence Typing [17], Single Nucleotide Polymorphism [18], Variable Number of Tandem Repeats [19] showed that PFGE remained the more discriminative method and is still considered as the “gold standard” for molecular epidemiology of P. aeruginosa [20]. This suggested that genetic changes in P. aeruginosa occurred by large rearrangements rather than by point mutations in housekeeping genes. Other genomotyping methods that also explored genomic rearrangements, such as rep-PCR, were slightly less discriminative than PFGE but have proved their efficiency for typing P. aeruginosa isolates in endemic or epidemic settings [21,22]. PCR-based approaches have the great advantage to be rapid, easy and cost-effective methods comparatively to PFGE [20].

The MDR1/2-ERIC1 clone could be considered as endemic and prevalent in the burns unit. Such resident multi-drug resistant strains have been previously reported [12,23]. In one case, the endemic strain evolved gradually from a moderate resistant to a multi-drug resistant phenotype [12]. Here, the resistant phenotype MDR1/2 appeared stably installed. However, we are not able to retrospectively perform the detection of ERIC1 genotype eventually associated with other antibiotic resistance patterns before 2005. A long-time persistent bacterial clone in a burn unit is submitted to the selective pressure imposed by the general use of topical antimicrobial agents. Owing to clinical evidence of low efficiency of local treatment upon wounds colonized with MDR1/2 clone, we undertook the in vitro testing of these strains regarding topical agents. As previously reported in a burn unit [12], we observed a decrease of susceptibility to SSD and SSDC of the isolates belonging to MDR1/2-ERIC1 clone. We also observed for two isolates that the low susceptibility to SSD and SSDC was not obligatory associated with the genomotype ERIC1 and/or / or with multi-resistance against antibiotics. In a recent study based on AWD assays, authors showed that 88% of non multidrug resistant

strains of the genera Acinetobacter, Pseudomonas, Klebsiella, Staphylococcus and Enterococcus were fully susceptible to topical agents compared to 80% of multi-drug resistant strains of the same genera [24]. We described for two pairs of strains isolated from the same patient (PAB49/55 and PAB27/28) rapid variation of their behaviour against antibiotics and/or topical agents. These variations could be explained by the co-existence of diverse sub-populations inside a same genomotype. Independent to / Regardless of their mechanism, the variations led to rapid adaptation in response to new selective pressures and probably according to the lowest energetic cost for the strain [25].

In spite of its use for 40 years ago, silver-sulphadiazine remains widely used today for topical antimicrobial treatment of burns [1]. Considered that / Because its antiseptic capabilities were not sufficient in all cases, a second mineral nitrate, cerium nitrate, has been added to SSD in the SSDC unguent. SSDC was shown to reduced infections as observed for SSD but also led to significant increase in survival rate of patients with a large percentage of total body surface area burned, even in presence of sepsis. According to the burn centre, / Among different burn centres, one observed 59% [9] and 39% [26] higher than expected survival rate when SSD and cerium nitrate were used in combination. It was generally recognized that / In general, cerium did not significantly enhanced the antimicrobial effect of SSD [27]. We confirmed here that the behaviour of P. aeruginosa against SSD and SSDC was similar in vitro. (Similar to what? Similar in response to both agents? Similar in vitro compared to results in vivo?) Therefore, the reduction in mortality rate might be attributed to the mechanic properties of SSDC that / SSCD, which forms a leather-like protective and soft / soft, protective, leather-like crust instead of the moist macerated eschar produced with SSD cream. SSD and SSDC were the more frequently used topical treatments in our unit since more than 95% of the patients entering the unit after thermal injuries were treated with Flammazine® (SSD) and/or Flammacerium® (SSDC). For patients with large burned surface, SSDC was used before excision and graft. The central place / role of SSD and SSDC in burn therapy, as well as the description of bacterial strains with reduced susceptibility to these agents urge the availability of efficient methods for their in vitro susceptibility testing.

Most topical antimicrobial efficacy studies in thermally injured patients are established in vivo in the Walker-Mason rat burn model, in which a bacterial strain is applied to a 20% scald burn with or without the tested topical agent [28]. This method could not be performed routinely. In vitro, diffusion methods for topical agents were proposed 30

years ago but did not encountered the success of the Kirby-Bauer method applied to antibiotics. However, most recent reports referring to diffusion methods for testing topical agents underlined that these methods were the simplest and the most reproducible [12, 24, 29]. The use of disks as support of the tested agents was not possible for all agents. Particularly for creams, unguents or gels such as SSD, SSDC or Betadine Gel®, well loading was obligatory. (new paragraph here)

For some authors, the correlation between in vitro testing and the clinical efficiency of topical agents is supposed to be low particularly because the in vitro assays explored bacteria in planktonic phenotype whereas the wounds are more likely to be colonized by bacteria with biofilm phenotype [30]. Considering this restriction, AWD assays with bacteria inoculated onto agar plates could present some advantages in comparison to methods using liquid broth. From a more general point of view, in vitro evaluation of bacterial susceptibility to topical agents and antiseptics suffer from the lack of standardization and defined cut-off values helping therapeutic decision. There are no specific tests for evaluating the efficacy of topical antimicrobials, including Minimal Inhibitory Concentration (MIC) (The abbreviation has been defined before so it should be used here without the full term.) determination, which have been standardized and approved by any oversight comity. Then, their use for the a priori prediction (Either “a priori” or “prediction”, but not both since they both mean the same thing.) of clinical efficiency, as done with antibiogram, should not be currently recommended. (new paragraph here)

Considering these limitations, we proposed (1) to undertake topical AWD assays on P. aeruginosa isolates owing to the preliminary evidence of low efficiency of local treatments, (2) to perform comparative analysis between the isolates of interest and unrelated P. aeruginosa strains. (Repetition of the statement of purpose. It may be useful at the beginning of the Discussion but is not useful in the middle of this section.) The inhibition diameters determined on a large reference population (The text should clarify that “reference population” refers to strains of bacteria and not to patients.) could be determined once and then used as a reference database. In semi-routine conditions, i.e. in response to a particular clinical situation, each clinical isolate should be tested in comparison with two strains of the reference population as controls. Moreover, the detection of MDR strains and/or endemic resident clone should lead to the determination of susceptibility to topical agents although these situations should not be strictly considered as pre-requisites before undertaking AWD assays. In vitro study of the mechanism of topical agent resistance should also be explored. (The

paragraph needs to be heavily edited. What are the authors trying to convince the readers of here?)

5 In our experience, the epidemic clone led to long-time wounds colonization and to refractory infections, suggesting (The text should make clear what the subject of “suggest” is.) the clinical significance of AWD assays on topical agents. Indeed, such long-time colonization and/or infection of burn wounds could be due to a less efficiency of SSD and SSDC. Unfortunately, precise clinical indicators could not be reported / determined in this retrospective study. Further studies are required to conclude about
10 the clinical significance of optimized comparative AWD assay on topical antimicrobial agents and about the benefice for the patients when this assay is performed in routine practice. (The paragraph needs to be heavily edited.)

COMPETING INTERESTS

15 The authors declare that they have no competing interests.

AUTHOR'S CONTRIBUTIONS

FA performed molecular experiments, coordinated AWD tests and analyzed data, FL is the principal clinical investigator and is / was involved in the manuscript drafting, /
20 drafting of the manuscript, SR participated to / in the study design and data acquisition, MD performed and interpreted AWD tests, HM interpreted results and revised the manuscript, MB is a clinical investigator involved in the critical analyse / analysis of results, / the results, FB design / designed and performed environmental investigations, / studies, SG performed and interpreted antibiotics / antibiotic testing, / tests, SP
25 designed the study and helped to draft the manuscript and EJB conceived and coordinated the study and write / wrote the manuscript. All authors read and approved the final manuscript.

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30 We are grateful to Jean-Luc Jeannot for his help in / with topical agents / agent manipulation. This study was partially supported by the association (*) ADEREMPHA (A translation into English of the names of institutions in non-English languages is helpful), Sauzet, France.

35 **References**

Legends to figures:

Figure 1: Selected ERIC-PCR profiles. The strains analyzed were PAB16, PAB27, PAB28, PAB40, PAB53, PAB61, PAB66, PAB67, PABH9 and PABH10 and were / as
5 indicated at the top of the gel. ERIC-PCR profiles were / are indicated at the bottom of the gel.

Figure 2: Repartition of the AWD diameter according / according to topical antimicrobial agents / agent and group of strains. Abbreviations of topical agents /
10 agent names as defined for / in table / Table 1. Group of strains as defined in the text. Inhibition zone diameters in mm; Bar, standard deviation.