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RESEARCH ARTICLE

Evaluating The Effectiveness of A Non-Locally Developed Commercial Phage Cocktail on Kenyan *Pseudomonas aeruginosa* Isolates

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ABSTRACT

Objectives: The use of bacteriophages (phages) as an alternative treatment for multidrug-resistant bacteria has recently gained popularity. Phage cocktails have been proposed for broad-spectrum therapeutic effects against such resistant bacteria. However, the effectiveness of non-locally formulated phage cocktails for therapy on Kenyan isolates has yet to be evaluated and is a subject of investigation. This study aimed to determine the *invitro* effectiveness of a non-locally made commercial pyophage cocktail on clinical *Pseudomonas aeruginosa* isolates from Kenya.

Methodology: Forty-nine *P. aeruginosa* isolates from Kenya were subjected to a pyophage cocktail for efficacy studies using direct spot test (DST) and efficiency of plating (EOP).

Results: The success rate of the cocktail was observed on 16.3% (8/49) isolates only and ineffective on 83.7% (41/49) isolates. Of the eight isolates that showed cross-reactivity from DST, six had complete lysis with a faintly hazy background. Five of these six isolates resulted in successful and high phage progeny production in plaquing efficiency (EOP ≥ 0.5).

Conclusion: Non-locally made commercial pyophage cocktail was ineffective against the 83.7% endemic clinical strains of the Kenyan *P. aeruginosa* isolates, demonstrating the importance of locally derived phage cocktails against endemic and multidrug-resistant isolates. *J Microbiol Infect Dis 2023; 12(4):25-30.*

Keywords: *Pyophage cocktail, P. aeruginosa, spot test, the efficiency of plating, host range*

INTRODUCTION

Pseudomonas aeruginosa is one of the disease-causing organisms associated with nosocomial infections in intensive care units (ICUs). It is an opportunistic Gram-negative organism with reported increased trends in antimicrobial resistance and worldwide dissemination of high-risk clones [1,2]. This organism is notorious for causing ventilator-associated pneumonia, urinary tract infections, post-operative infections from surgical and burn wounds, and bloodstream infections, particularly among hospitalized and immune-compromised patients [2]. Indeed, globally, *P. aeruginosa* infections account for high mortality proportions of 18-61% and are linked to 10-15% of hospital-associated infections [18]. The pathogenicity of *P. aeruginosa* isolates is triggered by their ability to acquire resistance to many classes of antibiotics and their intrinsic property of antibiotic resistance [19].

Current research in Kenya has reported the detection of pan-drug-resistant *P. aeruginosa* and high-risk strain types, which calls for urgent regulation, containment measures, and alternative treatment options [3]. Bacteriophages (phages) have been considered alternative therapeutic agents [4,5]. The exploitation of phages-based therapy was first introduced in 1915 but declined with the

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discoveries of antibiotics in the early 1940s [6]. Despite its promising results in treating bacterial infections, it was abandoned. However, with the advent of antibiotic resistance, phages have been sorted once again as alternative treatment options and have become popular since the 1980s [7]. In addition, phage cocktails have been purported to reduce the ability of bacteria to evolve phage resistance [8]. Phage cocktails comprise a mixture of phages possessing different host ranges from one or different Genera (polyphage), thus increasing the spectrum of activity as compared to a single type of phage (monophage) [8]. For instance, pyophage (PYO) is a polyvalent and commercially available phage cocktail in some parts of Europe and Russia. It was developed in Georgia at the Eliava Institute of Bacteriophages, Microbiology, and Virology and is used for soft tissue and skin infections, including pustule, nasal, pleural, abdominal, uterine, urinary bladder, and vaginal tissues [9].

Despite the promise of broad applicability, phage, and phage cocktail effectiveness could differ depending on the geographic locality of phage isolation phenotypic and genotypic variation of the bacterial strains used in phage isolation [10]. These observed variations could be attributed to the different antimicrobial profiles or the variation in the bacterial surface receptor [11,12] or through the ability of the bacteria to digest foreign genetic material by using restriction enzymes [10].

The success of phage cocktails, such as pyophage cocktail, in eradicating MDR *P. aeruginosa* infections and biofilms have been described in several European studies with a success rate of 87.9% to at least one of the tested and commercially available cocktails [10,13,14]. With the detection of global and high-risk strain types in Kenya, such phage cocktails could be applied since no viable treatment options are available. Furthermore, with the alarming trend of antibiotic resistance in disease-causing microbes globally, there is a need to evaluate the effectiveness of the available commercial phage cocktails. However, data on the effectiveness of commercially formulated phage cocktails from other countries on Kenyan local isolates needs to be included.

This research, therefore, aimed at assessing the efficiency of the Georgian-formulated pyophage (PYO) cocktail *invitro* on a collection of *P. aeruginosa* isolates from Kenya. Additionally, the cocktail's effectiveness was tested by comparing two methods of host range determination; the direct spot test method (DST) and the efficiency of plating (EOP).

METHODS

Ethical considerations and approvals

The study was accepted by the Institutional Research and Ethics Committee (ISERC) of the Institute of Primate Research (IPR/IRC/02/2022).

Pyophage (PYO) cocktail

The pyophage (PYO) cocktail #batch M1-801 is a commercial product developed at the Eliava Institute of Bacteriophage, Microbiology and Virology, Georgia [14]. However, it is commercially available only in the Europe Union and Russia. The sterile lysates of pyophage are effective on *Pseudomonas aeruginosa, Staphylococcus spp., Streptococcus spp, E. coli, Proteus mirabilis*, and *Proteus vulgaris*, with their titers ≥10^4 pfu/ml during formulation.

Bacteria resuscitation and subculture

Forty-nine isolates of *P. aeruginosa* (49 isolates; PA1-PA49) and *K. pneumoniae* (2 controls; KP1 and KP2) were acquired from the Center for Microbiology and Research, Kenya Medical Research Institute (CMR, KEMRI). These isolates were from patients with wounds and urinary tract infections from various hospitals in Nairobi, Kisii, Kisumu, Kilifi, and Kericho. Freezer stocks from -80 ºC stored in TSB with 40% glycerol were resuscitated through streaking on MacConkey agar (Sigma-Aldrich, Darmstadt, Germany), followed by incubation at 37 ºC for 24 hours. A single colony from the plate was inoculated into Tryptose Soy Broth (TSB) medium (Sigma-Aldrich, Darmstadt, Germany) and incubated at 37ºC in a shaker incubator at 200 rotation per minute (New Brunswick™ Innova® 40 Series Benchtop Incubator Shaker, Atkinson, New Hampshire, England) [15].

Screening for the potency of pyophage (PYO) cocktail

The effectiveness of the pyophage (PYO) cocktail against the *P. aeruginosa* was done through the spot test method as described by Clokie and Kropinski (2010), [16], and the

efficiency of plating as described by Kutter (2009), [17]. For the spot test, the overlay method of bacterial subculture was employed where 100 µl each of the 24hr-old broth bacterial culture was inoculated into molten soft agar (0.7%) with TSB (brought to equilibrate at 50 ºC) and dispensed on a square plate already plated with 1.5% Tryptose Soy Agar (TSA) (Sigma-Aldrich, Darmstadt, Germany). A transparent patched region following the overnight incubation was recorded as positive for pyophage cross-reactivity. Additionally, this positivity was scored numerically from 0-4, with 0 indicating no clearing/resistance, 1 for few countable plaques, 2 for considerable turbidity through the lysed region, 3 for clearing throughout but with indistinctly hazy background, and 4 with complete clearing [17].

All bacterial isolates that showed cross-reactivity with the phage cocktail were subjected to a more thorough assessment of productive phage infection through plating efficiency. Ten-fold serial dilutions of the pyophage lysate were briefly prepared in Saline Magnesium (SM) buffer with gelatin [5]. From the 10^{-1}-10^{-8} dilution series, 2 µL was dispended on a TSA plate overlaid with soft agar respective to the different host bacteria, then incubated overnight at 37 ºC. Clear regions on the bacterial lawn were recorded as positive for the phage lysis.

Calculation of phage titer and efficiency of plating

Plaque counts were done for plates with positive phaqe infection, as described in Clokie and Kropinski [16]. This was done in duplicate for each bacterial isolate and documented as the titer of the pyophage on the test isolates.

The plating efficiency was calculated according to Kutter [17]. The titer of our reference phage (pyophage) against *P. aeruginosa*, according to the manufacturer during formulation, was given to be 1.0×10^5 pfu/mL. However, the titer of the pyophage was not determined prior to this study due to the lack of the host strain used in phage propagation. An EOP ratio of 0.5 or more for a particular phage-bacterium combination was classified as "high production," an EOP ratio of 0.1 but below 0.5 was considered to be of "medium production," and a ratio between 0.001 and 0.1 was classified as “Low production” efficiency. An EOP ratio equal to or below 0.001 was termed inefficient.

Data analysis

The investigations were done in replicas, and average values were recorded using Microsoft Excel 2007 for Windows (Microsoft, Redmond, Washington, United States). EOP values obtained from each isolate were calculated and recorded. One-way analysis of variance (ANOVA) was used to determine significant differences at $p < 0.05$ using Statistical Package for Social Sciences (SPSS) software version 20 (SPSS Inc. Chicago, Illinois, USA).

RESULTS

Of the 49 isolates of *P. aeruginosa* isolates tested, only 16.3% (8/49) were lysed by the pyophage (PYO). Of these eight isolates, five attained a lysis score of 4, one isolate had a lysis score of 3, one isolate had a lysis score of 2, and one isolate had a lysis score of 1. The resistant rates of the Kenyan *P. aeruginosa* isolates were 83.7% (41/49 isolates)(Table 1).

In the plating efficiency of the pyophage (PYO) on the eight isolates of P. aeruginosa, an EOP of ≥ 0.5 was achieved in six isolates. In comparison, the remaining two isolates had an EOP of ≤0.5. Furthermore, five isolates with a lysis score of ≥3 resulted in high and successful productive infection. On the contrary, PA8 had a lysis score of 1 and achieved the highest progeny production (Table 1).

DISCUSSION

Our study is the first to report the pyophage (PYO) effectiveness in Sub-Saharan Africa, a different continent and geographical location from where the phage library was developed [14]. Furthermore, our results suggest that non-locally derived commercial phages are unsuitable for eradicating the Kenyan local *P. aeruginosa* bacterial isolates, indicating the importance of locally developed phage cocktails.

Overall, it was evident that the pyophage (PYO) cocktail had a narrow spectrum of activity with a success rate of 16.3% (9/49 isolates) and was ineffective against a majority of 83.7% (41/49). This shows that the pyophage needs to be sufficiently efficient in treating the endemic forty-one isolates of *P. aeruginosa*. The narrow spectrum of the pyophage could be attributed to restriction endonucleases, CRISPR mechanisms, or the cell surface receptors variation of the bacterial attributed to the broad and different geographical location of Europe,

where the isolates used in the cocktail formulation are phenotypically and genotypically varied as compared to the isolates in Kenya (Africa) [10,17]. These variations could be region-specific exposure to antimicrobial profiles or the variation in the bacterial surface receptors [11,12,20]. Additionally, the variations could be caused by the onset of phage resistance through the ability of the bacteria to digest extraneous DNA through restriction endonucleases activity or CRISPR [21,22].

Table 1: Effectiveness of pyophage cocktail on *P. aeruginosa* bacterial isolates in Kenya.

Bacterial isolate	Lysis score (spot test)	Phage titre (pfu/mL) on test strain	EOP = $\frac{Test\ strain\ titre}{Reference\ titre}$	EOP ratio interpretation
PA1	4	3.0×10^9	15	High production
PA2	4	5.0×10^3	0.05	Low production
PA3	4	2.0×10^5	2	High production
PA4	4	2.0×10^9	20,000	High production
PA5	4	2.0×10^5	2	High production
PA6	3	5.0×10^4	0.5	High production
PA7	2	5.0×10^3	0.05	Low production
PA8	1	1.5×10^6	30,000	High production

PA acronyms in the second column indicate the different isolates of *P. aeruginosa.* Pyo phage reference titer = 1.0×10^5 pfu/mL. Any EOP value ≥0.5 was scored as high production.

Table 1 Legend: The PYO-phage cocktail was lytic against eight isolates. Overall, six isolates had a lysis score of ≥3 from the spot test, and six resulted in high and successful progeny production in EOP.

Spot tests and plating efficiency are techniques for determining the phage host range [17]. In our study, the spot method resulted in completing lysis in five isolates (PA1-PA5), complete lysis with a faintly hazy background in one isolate (PA6), substantial turbidity in the cleared zone in one isolate (PA7), and few individual plaques in PA8. Complete lysis indicated that the phage effectively cleared that bacterial infection, while turbidity indicated the possibility of developing phage resistance. Furthermore, observation of individual plaques on the lytic zone shows that the phage titer was low in clearing the bacteria and hence can be optimized by increasing the phage titer.

Based on EOP analysis, we observed high phage production (≥0.5) in six isolates and low production (≤0.1) in two isolates (PA2 and PA7). The high phage production was indicative of high and desirable lytic efficiency, which ensures maximum and high production of new phage release [23], while low production was indicative of low lytic efficiency.

Even though the phage cocktail had complete lysis on two isolates (PA1 and PA2) in the spot test, it resulted in low progeny production in EOP. This is a scenario where the spot test overestimates the phage lytic activity. This overestimation could result from a high number of phages adsorbing to the bacterial cell membrane and lysing or lysis due to endolysins or bacteriocins in the phage stock [24]. Additionally, this 'lysis from without' could be attributed to adsorption resistance that causes a decreased interaction between phage and bacterium, resulting in phage death and bacterial survival or abortive infections, where both phage and bacterium die [25]. Similar reports of overrating the virulence action of spot tests compared to EOP have been observed [26].

On the other hand, the PA8 isolate had a few individual plaques on the spot test method but attained the highest plaquing efficiency in EOP. This could be explained by poor infection-initiation ability (receptors) combined with phage production of soluble factors that increased the likelihood of subsequent infection initiation [27].

Despite the cocktail having a narrow spectrum of activity, it resulted in high and successful productive phage infection on six of the eight isolates. According to Villarroel et al. (2017), the pyophage cocktail was initially formulated to contain roughly thirty distinctive phages targeting multiple different bacterial hosts in which the wide breadth of activity has been reported to lower the activity of a phage cocktail [14]. Instead, the present-day use of phage therapy recommends designing a phage combination between one and six phages targeting one strain of a bacterial species to eliminate cross-resistance [9,12]. Phages that target similar bacterial surface receptors evoke cross-resistance, with resistance mutations resulting in modification or loss of the shared receptor, preventing the adsorption of each phage [12,20].

The efficiency of the pyophage (PYO) cocktail in eradicating MDR *P. aeruginosa* isolates and eradicating biofilms has been described in numerous studies in Europe [13,28]. A study done in Turkey reported that out of ten isolates, nine were susceptible to the pyophage, while one was non-susceptible [29]. Additionally, a similar study in Turkey reported a success rate of 87.9% for at least one of the tested and commercially available phage formulations (Enko, Pyophage, SES, Fersisi, Intestiphage, and Staphylococcal Bacteriophage (Sb)) [28]. The close geographic proximities could explain this success between Turkey and Georgia and hence the lesser variation in the strain types that may have been used in the cocktail formulation compared to Kenyan strain types; thus, the disparity in the effectiveness of the pyophage cocktail.

This study has some limitations also. The cocktail phages evaluated were from Georgia; hence, the results may not be similar for all other commercial phages from other countries. Therefore, further studies on other commercial phage cocktails are recommended.

Conclusion

This study demonstrated that the *invitro* activity of phage formulations exploited for standard clinical practice in the Republic of Georgia was ineffective on most Kenyan *P. aeruginosa* clinical isolates. Therefore, this calls for Kenya's need to create a cocktail that is tailored and optimized against endemic and particularly multidrug-resistant isolates.

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All authors approved the study and submission. IJM, JN, and AN conceived the idea. IJM and MG conducted the experiment supervised by JN, EO, and AN. EO offered technical support. IJM and JN wrote the manuscript. EO, AN, and AK reviewed the manuscript.

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