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SHORT COMMUNICATION

Rummeliibacillus suwonensis: First Time Isolation from Human Feces by Culturomics

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Abstract

Gut microbiota is a complex ecosystem composed by trillions of microorganisms that are crucial for human health or disease status. Currently, there are two methodological options to explore its complexity: metagenomics and culturomics. Culturomics is an approach that uses multiple culture conditions (days of incubation, enrichment factors and growth temperature) and MALDI-TOF mass spectrometry for the identification of bacterial species and sequencing when this method fails. In this paper, we describe how Colturomic's protocol has allowed the first isolation in human sample of *Rummeliibacillus suwonensis*, a Gram positive, facultative anaerobe bacterium. The bacterium was isolated from feces of a 69 years old male with amyotrophic lateral sclerosis (ALS) recruited for a clinical trial assessing safety and efficacy of fecal microbiota transplantation in ALS. The first isolation of the microorganism dates back to 2013 from the soil of a South Korean mountain area. In this report, morphological description, biochemical characterization and antibiotic susceptibility tests were performed to outline the bacterial properties.

Introduction

The human body harbours trillions of microbial cells that coordinate fundamental actions for human life. The intestinal compartment is mostly inhabited by such microbial cell populations, which collectively form a real organ known as the gut microbiota [1]. Improving the knowledge on the gut microbial ecosystem in terms of composition and function represents a great interesting topic and exciting challenge for researchers. The advent of *omics* technologies has recently allowed to explore the diversity of the gut populations. Metagenomics describes the composition of complex environmental microbial ecosystem using next generation sequencing (NGS). On the other hand culturomics, in the last decade, investigated bacteria considered uncultivable using multiple culture conditions such as prolonged time of incubation, enrichment factors and growth temperature and finally MALDI-TOF mass spectrometry or gene 16S sequencing to identify bacterial species [1–4].

Currently, characterizing the intestinal microbiota represents a fundamental strategy to relate its possible role with the health of the individual. This aspect is proving to be essential not only in understanding the pathogenic mechanisms of intestinal diseases but also in systemic disorders. A

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concrete example is represented by diseases of the nervous system. In fact, it is now known how brain and gut continuously communicate through the so-called gut-brain axis in which intestinal bacteria play a key role. Currently, the efforts of researchers are focusing on understanding possible connections between gut bacteria and the nervous system specifically in highly debilitating diseases such as amyotrophic lateral sclerosis (ALS), a multi-system disease characterized primarily by progressive muscle weakness and cognitive dysfunction [5]. Study on mice have shown that gut microbiota acts by shaping immune tolerance and regulating the regulatory T cells number and suppressive function [5]. Culturomics may be a valid tool in typing gut microbiota of ALS patients to reveal potential correlations between bacterial species and disease features. Specifically, we are studying the ALS patients microbial pattern and in this paper, we describe the first human isolation of *Rummeliibacillus suwonensis.* The primary report on this genus dates back to 2009 when Vaishampayan et al. described two species: *Rummeliibacillus stabekisii* and *Rummeliibacillus pycnus* as a reclassification of *Bacillus pycnus* [6, 7]. In 2013, Her and Kim described a novel specie: *Rummeliibacillus suwonensis* (*R. suwonensis*) isolated from soil collected from a mountain area of South Korea [8]. The name *suwonensis* referring to the city of Suwon, South Korea where the strain was isolated while the name *Rummelii* is in honour of Dr John Rummel, a NASA astrobiologist. In general, the genus *Rummeliibacillus* belongs to the family Bacillaceae, to the order Bacillales, to the class Bacilli and to the phylum Firmicutes (Her 2013). *R. suwonensis* is a Gram-positive, spore-forming, motile rods, facultative anaerobe. The endospores are located terminally. Cells grow at an optimum of 37–45 ℃ and at NaCl concentrations of 0–5% [8].

Materials and Methods

Culturomics Protocol

The bacterial strain described above was isolated from the feces of a 69 years old male with amyotrophic lateral sclerosis (ALS) recruited for a clinical trial for testing the effects of fecal microbiota transplantation on ALS [5]. Bacterial strain was isolated by culturomics approach in the patient's feces sample collected at baseline (before treatment). In detail, thirty grams of fecal sample were suspended in 30 mL of saline solution. After the homogenization by Stomacher® 400 Circulator (SEWARD, UK), the fecal suspension is split in two aliquots and centrifuged at 3500×g for 10 min [9]. Supernatants were discarded and the two pellets were resuspended: one in 15 mL of Rumen fluid and the other one in 15 mL of supplemented Brucella Broth (BB, Remel INC., Lenexa, USA). Each enriched suspension (5 mL) was divided into six 2.5 mL aliquots, which were inoculated into a bottle of blood cultures for aerobes and anaerobes (Becton, Dickinson and Company, Benex Limited, Shannon, Ireland). Subsequently, the bottles were incubated at 30 °C, 37 °C and 42 °C for seven and fourteen days. After incubation period 10 µL of bacterial enriched suspension were plated on the following agar media and then incubated: TrypticSoy Agar (TSA, Becton Dickinson, Franklin Lakes, USA), Schaedler agar (SCH, Becton Dickinson, Franklin Lakes, USA), Columbia agar (CNA, Becton Dickinson, Franklin Lakes, USA) and Chocolate agar (PVX, Biomérieux, Marcy-l'Étoile, France). Culture conditions are summarized in Table 1.

Morphological Description, Strain Identification and Biochemical Properties

Bacterial identification was performed by MALDI-TOF mass spectrometry (Bruker Daltonics, Billerica, MA, USA). Each colony observed on agar plate was first isolated to obtain an axenic culture and subsequently spotted onto target plate and hydrated with 1 µl of α-cyrano-4-hydroxycinnamic acid (α-CHCA). Before each measurement, the instrument was calibrated using Bacterial Test Standard (BTS, Bruker Daltonics, Billerica, MA, USA). Scores greater than 1.9 are considered as an excellent identification rate [10, 11]. Staining methods, Gram and Schaeffer-Fulton (SF), were provided to describe microscopic morphology of bacterial cells. Specifically, the morphology of cells grown for three days at 37 °C was observed using an Olympus BX60 microscope (Olympus, Japan) at a final magnification of 1000x. SF method is based on the use of malachite green and safranine staining endospores in green and vegetative cells in red [12] (Fig. 1a). Moreover, biochemical tests on the isolated strain were performed. Further biochemical tests were performed by VITEK 2 using card GP (Biomérieux, Marcy-l'Étoile, France).

Antibiotic Susceptibility Testing

Considering the lack of data about *R. suwonensis* isolated from human sample, we investigated its antibiotic

Table 1 Culture conditions recommended in this study

	Temperatures condition		
	30 °C	37 °C	42 °C
Atmosphere condition			
Aerobiosis	TSA, CNA	0.5	TSA, CNA
Microaerophilic	PVX	PVX	PVX
Anaerobiosis	SCH, CNA	SCH, CNA	PVX

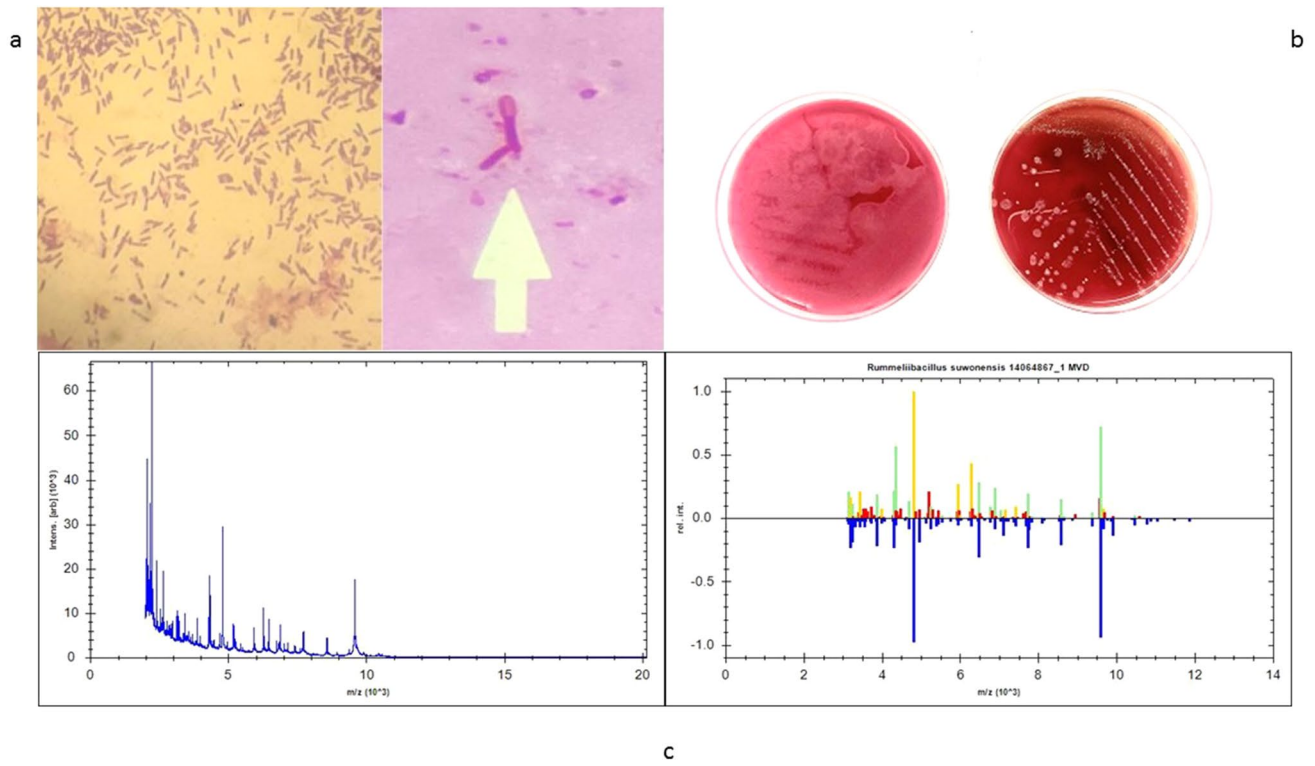

Fig. 1 **a** *R. suwonensis* on Gram staining (sx) and Schoeffler-Fulton staining (dx). **b** Colonies of *R. suwonensis* on TSA (sx) and SCH (dx). The endospores appears located terminally, **c** MALDI-TOF identification matching and strain spectrum

susceptibility pattern. According to the EUCAST breakpoint guidelines referred to *Bacillus* spp. [12], Epsilometer test (E-test) method for antimicrobial susceptibility testing was performed. Briefly, a 0.5 McFarland inoculum of bacterial suspension was streaked on a Mueller–Hinton agar plate (Biomerieux, Marcy-l'Étoile, France). MIC values were evaluated for the following molecules: meropenem, ciprofloxacin, vancomycin and erythromycin.

Results and Discussion

R. suwonensis was isolated by culturomics on a stool sample from an ALS patient recruited for a clinical trial of Fecal Microbiota Transplantation in ALS. Among the key players in the pathogenesis of ALS, microglia and T regulatory lymphocytes (T-reg) are candidate cells for modifying the course of the disease. The gut microbiota acts by shaping immune tolerance and regulating the T-reg number and suppressive function, besides circulating neuropeptides, and other immune cells that play in concert through the gut-brain axis [5].Specifically, after three days by the isolation, colonies of *R. suwonensis* have grown at 37 °C in aerobic and anaerobic condition, showing different characteristics. In aerobic condition, on TSA agar, colonies were 0.2–0.5 cm in diameter, approximately circular, undulate, raised and greyish. Whereas in anaerobic conditions, on SCH agar, colonies appeared greyish, large, flat and dry tending to occupy the entire space of the plate (Fig. 1b). The accurate MALDI-TOF identification showed a clear spectrum and high identification score of 2.1 (Fig. 1c). Biochemical analyses were performed to characterize the strain enzyme pattern, resulting catalase positive and oxidase positive. Table 2 shows the biochemical properties of the isolated strain. Moreover, staining preparation was provided to obtain further morphology features. Specifically, bacteria showed a Gram-positive stain and the presence of endospore located terminally confirming the characterization made by Her and Kim in 2013 [8]. Furthermore, antibiotic susceptibility test was carried out to highlight any antibiotic resistance. Epsilon test was performed considering EUCAST breakpoint for Bacillus spp. (excepted *Bacillus anthracis*). In Table 3 are listed the four main drugs used for Bacillus [13, 14]. Meropenem, vancomycin and erythromycin showed a susceptibility profile while ciprofloxacin an intermedium profile. In conclusion, the culturomics analysis proved to be a useful methodological option for the isolation of new species or species classically considered as uncultivable, although it is time-consuming and unsuitable for standard clinical microbiology. Nevertheless, environmental

Table 2 Biochemical profile of *R. suwonensis*. GP card includes test for the following reactions:

AMY	+	PIPLC	–	AspA	–	AGAL	–	dMAL	–
APPA	+	CDEX	–	BGURr	–	URE	–	MBdG	+
LeuA	–	ProA	–	dSOR	–	NAG	+	dTRE	+
AlaA	–	TyrA	–	LAC	–	dMNE	+	AGLU	–
dRIB	–	lLATk	–	dMAN	–	SAC	+	PHOS	–
NOVO	–	NC6.5	–	SAL	-	BGAL	–	BGUR	–
dRAF	+	O129R	–	ADH1	–	AMAN	–	dGAL	–
OPTO	–	dXYL	+	BGAR	–	PyrA	–	BACI	–
PUL	+	ADH2s	+	POLYB	–				

Phosphatidylinositol phospholipase C (PIPLC), arginine dihydrolase (ADH1,ADH2s) (two tests), α-glucosidase (AGLU), alanine-phenylalanine-proline arylamidase (APPA), l-aspartic acid arylamidase (AspA), β-galactosidase (BGAL), α-mannosidase (AMAN), alkaline phosphatase (PHOS), l-leucine arylamidase (LeuA), proline arylamidase (ProA), β-glucuronidase (dGAL) (two tests), α-galactosidase (AGAL), l-pyroglutamic acid arylamidase (PyrA), alanine arylamidase (AlaA), tyrosine arylamidase (TyrA), and urease (URE). The GP identification card also tests acid production from the following substrates: amygdalin (AMY), xylose (dXYL), α-cyclodextrin (CDEX), sorbitol (dSOR), galactose, ribose (dRIB), lactate, lactose (LAC), N-acetyl-glucosamine (NAG), maltose (dMAL), mannitol (dMAN), mannose (dMNE), methyl-β-d-glucopyranoside (MBdG), pullulan (PUL), raffinose (dRAF), salicin (SAL), sucrose (SAC), trehalose (dTRE)

Table 3 Antibiotic susceptibility pattern of *R. suwonensis*

Antimicrobial agent	EUCAST breakpoint *(mg/L)		MIC (mg/L)	Interpretation
	S ≤	R >		
Meropenem	0.25	0.25	0.25	S
Ciprofloxacin	0.001	0.5	0.023	I
Vancomycin	2	2	0.125	S
Erythromycin	0.5	0.5	0.016	S

*Clinical breakpoint are referred to *Bacillus* spp. excepted *Bacillus anthracis*

microbiologists have continued to develop empirical culture strategies playing on temperature, atmosphere and then allowing to extend the list of cultured microorganisms. New culture conditions and improved identification methods lead to an increase of bacterial species from 1791 in 1980 to more than 12,000 in 2013 [15]. There are many examples of culturomics' study designs on gut microbiota. In fact, stool samples from healthy people or from patients with intestinal or extra-intestinal disorders represent the "golden source" for the isolation of new species. In the last decade, literature is booming of papers about new species with specific functions potentially correlated to the healthy/disease status. In 2007 a Bacteroides strain defined D8, with high capacity to reduce luminal cholesterol to coprostanol, was isolated from feces of a male volunteer [16]. Moreover, in 2013 from a single anorexia nervosa stool sample, Pfleiderer and collaborators identified 11 new bacterial species [15]

Conclusions

The aim of this paper was to underline the usefullness of Culturomics as a powerful approach in gut microbiota typing to intrigue more researchers in this field. Moreover, in the "personalized medicine era", understanding the potential impact of the bacteria–host relationship is one of the main aims for researchers. Once this correlation has been identified, the main challenge will be intervening on the intestinal microbiota to intervene on its composition and, consequently, the functional outputs. In this scenario, the isolation of new hardly cultivable bacteria such as *Rummeliibacillus suwonensis* expands the spectrum of newly identified species and underlines the role of studies on human feces to understand the possible connection between unusual isolated species and clinical or geographical status. At present in our clinical trial on ALS patients, this strain was detected in fecal sample of one patient. Obviously, further microbiota characterization studies should be conducted in healthy patients and ALS patients to confirm or disprove the potential association between microbial species and disease.

Author Contributions GQ, AG and LM contributed to the study conception, design and writing. GF and SB performed material preparation and data collection. JM, CS, GC and MS contributed in patient's recruiting. AA, EN and GN contributed in laboratory activities. LM was the supervisor in writing and corrections.

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Data Availability Yes.

Code Availability Not applicable.

Declarations

Conflict of interest The authors declare no competing interest.

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Informed Consent Yes.

Consent for Publication Yes.

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