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Gianfranco Donelli *Editor*

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Personalized Nutrition for Microbiota Correction and Metabolism Restore in Type 2 Diabetes Mellitus Patients

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Abstract

Type 2 diabetes is one of the most common noncommunicable diseases in the world. Recent studies suggest a link between type 2 diabetes and microbiota, as well as the ability to treat and prevent it using personalized approaches to nutrition. In this work, we conducted clinical studies on the effects of a personalized diet on 56 female patients. Biochemical, physical, and immunological parameters were measured by standard methods on days 1 and 18 of the experiment. Gut and oral microbiota studies were performed in dynamics on days 1, 7, 11, and 18 using real-time polymerase chain reaction. With the help of the developed information system, a personalized diet was developed for each participant of the experiment. In the group of patients following personalized diets a statistically significant decreasing levels of glucose, thymol test, creatinine, very low-density lipoprotein, urea, secretory IgA, and tumour necrosis factor-α, and improvement in all physical parameters were observed. There was a statistically significant increase in uric acid, sodium, and magnesium. Statistically significant changes in gut microbiota were observed in *Enterococcus faecalis*, *Escherichia coli* (lac+, lac−), *Lactobacillus* spp., and *Candida* spp. Such microorganisms of oral microbiota as *E. faecalis*, *Lactobacillus* spp., *Pseudomonas aeruginosa*, and *Candida* spp. demonstrated statistically significant changes. All these changes indicate an improvement in the patients' condition in the experimental group compared to the control group. Our algorithm used for the development of personalized diets for patients with diabetes type 2 demonstrated clinical efficacy of its implementation.

Keywords

Human microbiota · Metabolism regulation · Noncommunicable disease · Personalised diet · Prognostic correction

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1 Introduction

Type 2 diabetes mellitus (T2D) is a growing global health problem closely related to the epidemic of obesity. It is characterized by dysregulation of carbohydrate, lipid, and protein metabolism and results from impaired insulin secretion, insulin resistance, or a combination of both (DeFronzo et al. 2015). T2D is one of the noncommunicable diseases (NCDs) common among almost all people in the world (Raychaudhuri 2011) regardless of their age and region due to the changes in lifestyles, genetics, and environmental factors, all of which together influence the disorder (Raj et al. 2018).

Typical clinical markers of type 2 diabetes include glucose and glycosylated haemoglobin, increased cholesterol, triglycerides, low-density lipoprotein, very low-density lipoprotein, and decreased high-density lipoprotein (Krauss 2004). Metabolic parameters such as urea, uric acid, creatinine, bilirubin, calcium, magnesium, sodium, alanine aminotransferase, and others involved in lipid profile regulation are an additional source of complete information about the biochemical status of the human body. Recent researches have demonstrated that the development of low-grade inflammation is a consequence of gut microbiota alteration, which is closely related to metabolic disorders such as obesity and T2D (Cani et al. 2012; Minihane et al. 2015). In particular, in the majority of patients suffering from diabetes the levels of *Bifidobacterium* and *Lactobacillus* decrease, which leads to an increase in the levels of *Bacteroides, Prevotella, Peptococcus, Clostridium, Proteus, Staphylococcus,* and *Candida*. Importantly, T2D subjects have smaller amounts of butyrate producing bacteria, such as *Roseburia intestinalis* and *Faecalibacterium prausnitzii*, and a mucus-degrading bacterium *Akkermansia muciniphila* (Tilg and Moschen 2014).

Studies conducted within the "Human Microbiome" project (Group et al. 2009) demonstrated that intestinal microbiome can be dominated by different ratios of beneficial microorganisms and still perform identical functions. Thus, it is not only the species composition of the microbiome, but also its "function" that is important. Herewith, it is obvious that the microbiome of each individual is unique.

Recently, numerous research studies have been conducted to find a relationship between nutrition and its impact on human health. Nevertheless, today a balanced diet principle remains practically unapplied. The reason is, on the one hand, that people misunderstand (underestimate) the role of food as a source of essential balanced nutrients. On the other hand, there are huge amounts of data on "proper nutrition" (rational nutrition) available and they are often contradictory, scientifically unsubstantiated, and clinically unconfirmed. A new modern challenge is the use of P4 (predictive, preventive, personalised, and participatory) approaches, in particular personalized nutrition, in medical practice.

The diet-microbiome interplay is currently the basis for personalized nutrition introduction and microbiota composition is the key factor affecting responsiveness to nutritional interventions that will soon take into account initial stratification of individuals on the basis of microbiota (Ercolini and Fogliano 2018).

The health benefits of adherence to the Mediterranean diet, as well as the relationship between microbiota and its associated metabolome in people consuming varied diets ranging from vegan to omnivorous, are now evidence-based (Shanahan et al. 2017).

In our opinion, the most promising way of individual microbiome correction, as well as prognostic modulation of local immune response, is the use of complete personalized diets rather than individual components. The most popular diets whose positive health effects on the human body are considered to be established include the Mediterranean diet, vegetarian/vegan diet, high-fibre diet, and high-protein diet.

The antioxidant and anti-inflammatory effects of the Mediterranean diet on the whole as well as the effects of this diet's individual components, in particular olive oil, fruits and vegetables, whole grains, and fish, have a beneficial impact on abdominal obesity, lipids levels, glucose metabolism, and blood pressure levels (Kastorini et al.

2011). Gut microbiota in individuals following the Mediterranean diet is characterized by high levels of *Lactobacillus* spp., *Bifidobacterium* spp., and *Prevotella* spp. and low levels of *Clostridium* spp., which relates to weight loss, improvement of the lipid profile, and decreased inflammation (Singh et al. 2017).

For vegetarians and vegans, the most relevant risk factors for chronic disease, such as body mass index (BMI), lipid variables, and fasting glucose, are significantly lower. People following a plant-based dietary pattern demonstrate significantly lower levels of BMI, total cholesterol, LDL-cholesterol, triglycerides, and blood glucose when vegetarians were compared to nonvegetarians, and lower levels of BMI, total cholesterol, and LDL-cholesterol when vegans were compared to nonvegans (Dinu et al. 2017). People following vegan and vegetarian diets rich in fermentable plant-based foods were reported to have a microbiota characterized by a lower abundance of *Bacteroides* spp. and *Bifidobacterium* spp. (Wu et al. 2016).

High fibre intake is associated with lower serum cholesterol concentrations, lower risk of coronary heart disease, reduced blood pressure, enhanced weight control, better glycaemic control, reduced risk of certain forms of cancer, and improved gastrointestinal function (Anderson et al. 2009). One study revealed that three diets containing different fibre-rich whole grains (barley, brown rice, or a combination of both) increased microbial diversity, the Firmicutes/Bacteroidetes ratio, and the abundance of the genus *Blautia* in faecal samples (Oriach et al. 2016).

High-protein diet decreases weight, fasting glucose, and insulin concentrations as well as total and abdominal fat. In addition, this diet significantly decreases LDL cholesterol concentrations (Parker et al. 2002). Dietary protein intake in humans has been associated with the *Bacteroides* enterotype (Oriach et al. 2016).

In previous studies, we obtained data demonstrating that extracts of certain edible plants rich in biologically active substances (BAS) specifically stimulate the immune response and have anti-inflammatory properties. We also proved that these extracts are able to specifically modulate intestinal microbiota (Bati and Boyko 2013).

In our previous studies involving different mouse models, we showed the molecular mechanism by which different gut commensal representatives modulate local immune response at mucosal sites in a strain- or species-specific manner. We were able to analyse *in vitro* the effects of individual commensal bacteria on human monocyte-derived dendritic cells (moDCs)-mediated inflammation and effector T-lymphocyte priming conditions mimicking unique intestinal microenvironment. Human moDCs expressing peroxisome proliferator-activated receptor gamma (PPARγ) also regulate cell surface expression of type I and II CD1 glycoprotein receptors as well as mucosa-associated CD103 protein differently in the absence or presence of all-trans-retinoic acid (ATRA), when ATRA provides a tolerogenic effect. In other words, this makes the pro- and anti-inflammatory reprogramming of this population of immune cells possible (Bene et al. 2017).

However, applying all these observations in practice taking into consideration patients' microbiome uniqueness is a challenge.

Additionally, it is known that the geographical location of plant food ingredients' growth affects the quantitative and qualitative composition of their BAS. Also, geographical location determines people's lifestyles, their habits and traditions, and diets.

Previously, within the BaSeFood project, we conducted a study of priority dishes in the Black Sea region, including Ukrainian ones. We determined the nutritional value and composition of food products, which formed the basis for the creation of the First National Composite Database of Food (Costa et al. 2013). One of the tasks of this work was to investigate the fundamental possibility of creating or developing personalized (individual) approaches (diet plans) using traditional dishes (based on traditional dishes) of our region as a source of BAS selected for their known biological effects on the microbiome and local immune response and that could be used to treat T2D in a controlled diet study (Danesi et al. 2013; Pallah et al. 2019).

Following to numerous *in vitro* studies (Pallah et al. 2019; Bati and Boyko 2016, 2017) and based on *in vivo* experiments data about main influences of various plant originated compounds and defined beneficial lactic acid bacteria (LAB) strains on gut microbiota, mucosal immune response and lipid metabolism of tested mice and rats (Bati and Boyko 2016; Meleshko et al. 2020) the selection procedure of most promising ethnical foods had been performed.

Thus, the aim of this study was to investigate the possibility of correction of lipid metabolism of patients with T2D using a personalised diet based on the most important microbial, biochemical, and immunological biomarkers of chronic inflammation.

To achieve this goal, we focused on lipid metabolism, immune, and microbiome biomarkers as a whole, as well as patients' individual characteristics (differences), to be able to regulate those indices that are considered major evidence-based determinants of T2D.

2 Materials and Methods

Patients of the Mukachevo Central District Hospital, Therapy Department, took part in the controlled clinical trial; all participants gave written informed consent.

Women aged 39–68 years with T2D were selected according to the criteria typical of this nosology (DeFronzo et al. 2015). Exclusionary criteria involved smoking, alcohol or drug abuse, pregnancy, and unstable medical status. No participants had clinically significant cardiovascular, renal or liver disease, a history of cancer or any other comorbidities. Patients who participated in the study did not take any other drugs.

Eligibility requirements were fulfilled and enrolment procedures were performed in accordance with the EU Clinical Trials Regulation (Regulation (EU) No 536/2014). The study protocol was approved by the Uzhhorod National University, Research Ethics Committee.

To confirm the effectiveness of personalized diet plans, a randomized controlled trial was conducted in two parallel groups. Group I (experimental one) included patients who followed an 18-day personalized diet, which included individually selected products rich in BAS and yogurts with unique microbial starters. Group II (control one) involved patients who, for 18 days, ate berries and yogurt prepared without microbial starters in the morning. Patients were not instructed to do additional physical exercise. The experimental group consisted of 35 patients and the control one of 21 patients. The study lasted for roughly a month. Before and after the diet course we measured five groups of parameters (total 62 parameters): (1) patients' biochemical status; (2) gut microbiota; (3) oral microbiota; (4) immune status; and (5) physical parameters of patients (measurement of body weight, circumference of waist, thighs, and upper thighs). Gut and oral microbiota studies were performed in dynamics on days 1, 7, 11 and 18 of the experiment.

In order to conduct measurements, that is to determine the condition (severity and course of the disease), for each individual we identified typical to this disease diagnostic markers for the detection of T2D, such as blood glucose, lipid profile (cholesterol, LDL, HDL, VLDL, triglycerides, and atherogenicity levels), glycosylated haemoglobin, total protein, and bilirubin levels, as well as typical diagnostic enzymes (amylase, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, gamma-glutamyl transferase, and total creatine kinase), thymol test, and indicators measuring the state of the excretory system (albumin, urea and uric acid, and total creatinine) and micro- (iron) and macronutrient (potassium, magnesium, calcium, sodium) blood composition, as an evidence of existing metabolic disorders. Regarding immune parameters, we limited ourselves to the well-known indicators of inflammatory processes, that is markers of inflammation and their agonists (IL-1β, Il-10, TNF-α). However, we also considered previously identified (selected) local inflammation markers, such as levels of total and secretory immunoglobulin A in serum (IgA, SIgA). During the study of intestinal and oral microbiome we focused on

such target groups of microorganisms as (1) typical intestinal commensals and the so-called beneficial microorganisms (Enterobacteriaceae family, genera *Staphylococcus, Streptococcus*, *Lactobacillus, Candida, Clostridium* spp.); (2) opportunistic microorganisms - *Pseudomonas aeruginosa, E. faecalis, Staphylococcus* spp., Enterobacteriaceae; (3) markers of metabolic disorders that we identified earlier (*E. coli* lac+, *E. coli* lac-, *Bifidobacterium* spp., *Enterococcus* spp.) (Petrov and Boyko 2014).

Blood formula (red and white blood cells, monocytes, lymphocytes, platelet assay, and eosinophils) was identified using Mythic 22 Orphee S.A. (Switzerland) Haematology system. Erythrocyte sedimentation rate (ESR) was measured using the Westergren method. Haemoglobin was identified calorimetrically. All biochemical parameters were assayed using Cobas c 311 (Roche/Hitachi) Switzerland.

Intestinal and oral microbes were studied according to our own method using the following nutrient media: Mitis Salivarius Agar, Bile Esculin Agar, Mannitol Salt Agar, Endo Agar, Bismuth Sulphite Agar, HiCrome Clostridial Agar, Sabouraud Dextrose Agar, Lactobacillus MRS Agar, Bifidobacterium Agar, Bacteroides bile esculin agar, Propionibacter Isolation Agar, L.D. Esculin HiVegTM Agar (manufactured by HiMedia Laboratories, India), UriSelect™ 4 Medium (Bio-Rad Laboratories, Inc., USA), and Blaurock semi-liquid modified hepatic medium (manufactured by Liofilchem, Italy). Identification of isolated microorganisms was performed using biochemical test systems ANAERO-23, ENTERO-24, NEFERM-test, Candida-23, STAPHY-16, and STREPTO test 24 (Erba Lachema s.r.o., Czech Republic). Microbiome studies were also performed using real-time polymerase chain reaction (qPCR). Immune parameters were measured using indicator immunosorbent systems Vector-Best (Russian Federation); results were read at a wavelength of 450 nm using a plate immunosorbent assay BioTek Elx800.

With the help of the developed information system and created an algorithm based on linear programming approaches, which allows selecting food for any individual (patient) in accordance with the state of her gut microbiota and immune and biochemical parameters, a personalized diet was developed for each participant of the experiment. Developed diets included products that contain functioning groups of biologically active substances such as polyphenols, anthocyanins, and flavonoids as well as unique microbial starters for fermentation. Sequenced strains of *Lactobacillus casei* IMB B-7412, *Lactobacillus plantarum* IMB B-7414, and *Lactobacillus plantarum* IMB B-7413 were used to prepare yogurts. The selection of food products was based on WHO recommendations (https://www.who.int/nutrition/publications/nutrient/en/), taking into account individual wishes and contraindications, as well as when determining the portion size - individual characteristics of patients such as the level of physical activity, body mass index, etc.

Statistical analyses were performed using the statistical program GraphPad Prism version 3.00 (GraphPad Software, USA). All data are presented as the mean ± SD or mean ± SE. For normally distributed data, checked used Shapiro-Wilk test, comparisons were tested using ANOVA. The two-tailed Mann-Whitney U-test was used for comparisons between the groups. P values <0.05 were considered statistically significant.

3 Results

On the first day of the experiment, in all patients diagnosed with type 2 diabetes there was an increase in the level of biochemical parameters observed: glucose (the real average value is 8 times higher than the allowed excess of the average value of the norm), LDH (the real average value is 5 times higher than the allowed excess of the average value of the norm), HbA1C (the real average value is 1,5 times higher than the allowed excess of the average value of the norm), and immunological indicator IL-10 (the real average value is 2 times higher than the allowed excess of the average value of the norm), as well as physical parameters such as BMI (the

real average value is 5,5 times higher than the allowed excess of the average value of the norm). Also, a decrease in HDL levels was observed: the real average value is 1.5 times lower than the allowed decrease in the average value of the norm (see Table 1, Fig. 1).

The composition of the intestinal microbiota on day 1 of the experiment demonstrated a predominance of enterococci and lactobacilli with a significant variety of commensal and opportunistic microorganisms, namely enterobacteria, pseudomonads, streptococci, staphylococci, bacilli, and candida. We observed an increase level of *E. faecalis* (the real average value is 2 times higher than the allowed excess of the average value of the norm), a decrease in levels of *E. coli* (lac+) (the real average value is more than 8 times lower than the allowed decrease in the average value of the norm), *E. coli* (lac−) (the real average value is 4 times lower than the allowed decrease in the average value of the norm) and *Lactobacillus* spp. (the real average value is more than 1,5 times lower than the allowed decrease in the average value of the norm) (see Figs. 2 and 3). The oral microbiota was characterized by a predominance of lactobacilli, enterococci, and streptococci, as well as a number of other bacteria, such as *E. coli* (lac+), *Citrobacter* spp., *E. cloacae*, *P. aeruginosa*, *S. epidermidis*, *Bacillus* spp., and *Candida* spp. We observed an increased level of *E. faecalis* (the real average value is 4 times higher than the allowed excess of the average value of the norm), *Lactobacillus* spp. (the real average value is 7,5 times higher than the allowed excess of the average value of the norm), *P. aeruginosa* and *Candida* spp. (the real average value is 2 times higher than the allowed excess of the average value of the norm) (see Figs. 2 and 4). On the first day of the experiment, no statistically significant difference was observed between the control and experimental groups.

After 18 days of the experiment, no statistically significant changes in parameters were observed in the control group, but there were changes in blood and physical parameters and microbiota composition in the experimental group. According to the data obtained, there was a decrease in the levels of such biochemical parameters as glucose, bilirubin, thymol test, cholesterol, HDL, LDL, VLDL, iron, gamma-glutamyl transferase, total protein, urea, creatinine, LDH, HbA1C, and triglycerides as well as changes in all immune and physical parameters. Also, an increase in amylase, alkaline phosphatase, calcium, creatine kinase, aspartate transferase, alanine aminotransferase, uric acid, sodium, magnesium, albumin, and atherogenicity levels was observed. Herewith, on day 18 of the experiment all indicators were almost unchanged in the control group (see Table 1).

After adherence to a personalized diet, in the experimental group patients there was a statistically significant reduction in the following parameters: glucose, thymol test, VLDL, urea, creatinine, sIgA, and TNF-a, as well as all physical parameters. There was a statistically significant increase in such biochemical parameters as uric acid, sodium, and magnesium. Regarding intestinal microbiota indicators, there was a decrease in the levels of all microbiota members except lactobacilli. Statistically significant changes were observed in *Enterococcus faecalis, Escherichia coli* (lac+), *Escherichia coli* (lac−), *Lactobacillus* spp., and *Candida* spp. The oral microbiota was characterized by a decrease in the number of all representatives except lactobacilli. Such microorganisms as *E. faecalis, Lactobacillus* spp., *P. aeruginosa,* and *Candida* spp. demonstrated statistically significant changes.

Statistically significant changes in the concentration of microorganisms (in dynamics) were observed in both the intestinal microbiota (*E. faecalis, E. coli* (lac+), *E. coli* (lac−), *Lactobacillus* spp., and *Candida* spp.) and oral microbiota (*E. faecalis, Lactobacillus* spp., *P. aeruginosa,* and *Candida* spp.) (see Figs. 5 and 6). Dynamic intestinal microbiota changes in the experimental group (see Fig. 4) demonstrate that the average concentration of *E. faecalis* remained unchanged until day 11 while a statistically significant difference compared to the first day appeared on day 11 and the tendency to a decrease remained on day 18 of the experiment. *E. coli* (lac+) is characterized by a decrease in concentration throughout the

Table 1 Measured parameters in experiment

Parameter, units	Experimental group, mean ± SD		Control group, mean ± SD		Reference range
	Day 1	Day 18	Day 1	Day 18	
Blood parameters					
Amylase, u/l 37°C	42.076 ± 2.133	46.076 ± 3.789	42.302 ± 1.204	42.056 ± 1.002	< 90
Alkaline phosphatase, u/l 37°C	59.990 ± 2.423	63.004 ± 5.841	59.684 ± 1.528	59.447 ± 1.472	35–104
Bilirubin, Mol/l	17.141 ± 2.849	16.774 ± 2.295	16.622 ± 1.499	16.493 ± 1.854	< 21
Glucose, mmol/l	12.316 ± 2.186[a]	9.961 ± 2.063[a]	12.187 ± 1.32	11.812 ± 1.257	4.1–5.9
Calcium, Mol/l	2.451 ± 0.038	2.530 ± 0.081	2.458 ± 0.02	2.462 ± 0.027	2.25–2.75
Thymol test, u/l	3.219 ± 0.592[a]	2.229 ± 0.601[a]	3.214 ± 0.389	3.173 ± 0.328	0–4
Cholesterol, mmol/l	5.427 ± 0.913	5.055 ± 0.581	5.454 ± 0.52	5.499 ± 0.487	2.9–5.2
HDL cholesterol, mmol/l	1.378 ± 0.233	1.314 ± 0.242	1.404 ± 0.134	1.411 ± 0.119	> 1.68
LDL cholesterol, mmol/l	3.383 ± 0.727	2.933 ± 0.398	3.382 ± 0.422	3.413 ± 0.39	< 3.34
VLDL, mmol/l	0.967 ± 0.233[a]	0.781 ± 0.284[a]	0.958 ± 0.161	0.942 ± 0.149	0.26–1.04
Creatine kinase, mmol/l	66.994 ± 15.466	73.219 ± 18.382	66.794 ± 9.489	67.681 ± 8.507	26–192
Iron, μmol/l	15.789 ± 2.915	15.464 ± 1.852	15.312 ± 1.578	15.059 ± 1.893	8.95–30.43
Gamma-glutamyl transferase, u/l 37°C	31.210 ± 5.256	24.747 ± 6.33	30.825 ± 3.197	32.512 ± 2.661	6–42
Aspartate transferase, u/l 37°C	17.415 ± 2.547	20.646 ± 4.87	17.377 ± 1.573	17.196 ± 1.52	< 32
Alanine aminotransferase, u/l 37°C	21.159 ± 3.846	27.396 ± 7.653	20.921 ± 2.23	20.877 ± 2.249	< 32
Total protein, g/l	67.829 ± 2.834	67.381 ± 1.186	68.126 ± 1.294	68.559 ± 1.443	66–87
Urea, mmol/l	5.558 ± 0.715[a]	4.530 ± 0.716[a]	5.583 ± 0.451	5.754 ± 0.404	2.76–8.07
Uric acid, μmol/l	265.097 ± 45.594[a]	290.966 ± 51.233[a]	263.386 ± 26.167	274.948 ± 19.444	150–350
Potassium, Mol/l	4.409 ± 0.202	4.410 ± 0.138	4.438 ± 0.097	4.477 ± 0.12	3.5–5.5
Creatinine, μmol/l	76.980 ± 4.462[a]	68.290 ± 3.662[a]	77.808 ± 2.18	78.289 ± 2.601	45–84
LDH, u/l 37°C	366.829 ± 53.892	344.897 ± 46.811	367.829 ± 33.487	383.114 ± 29.803	135–214
Sodium, mmol/l	136.181 ± 2.488[a]	139.41 ± 1.204[a]	136.496 ± 1.469	136.631 ± 1.822	132–145
Glycosylated haemoglobin, %	8.892 ± 0.731	8.235 ± 0.956	8.866 ± 0.395	8.782 ± 0.468	< 7.0
Magnesium, mmol/l	0.701 ± 0.08[a]	0.767 ± 0.058[a]	0.717 ± 0.036	0.726 ± 0.049	0.66–1.07
Albumin, g/l	44.467 ± 1.671	45.021 ± 0.898	44.612 ± 0.865	44.828 ± 0.932	35–52
Atherogenic coefficient, mmol/l	2.842 ± 0.52	2.975 ± 0.626	2.798 ± 0.314	2.796 ± 0.31	< 3.0
Triglycerides, mmol/l	2.118 ± 0.509	1.747 ± 0.664	2.099 ± 0.351	2.064 ± 0.324	< 2.26
Immune parameters					
IgA, g/l	3.187 ± 0.871	2.624 ± 0.44	3.113 ± 0.492	2.877 ± 0.481	0.7–4.0
sIgA, mg/l	4.636 ± 1.572[a]	3.399 ± 1.215[a]	4.675 ± 0.997	4.29 ± 0.885	1.60–5.48

(continued)

Table 1 (continued)

Parameter, units	Experimental group, mean ± SD		Control group, mean ± SD		Reference range
	Day 1	Day 18	Day 1	Day 18	
IL-10, pg/ml	13.783 ± 3.328	11.408 ± 2.147	13.936 ± 1.958	13.262 ± 1.999	< 9.1
TNF-a, pg/ml	7.06 ± 0.988[a]	0.154 ± 0.181[a]	6.968 ± 0.554	7.048 ± 0.519	< 8.1
IL-1b, pg/ml	3.018 ± 1.312	2.483 ± 1.716	3.21 ± 0.697	2.97 ± 0.75	< 5.1
Physical parameters					
Weight, kg	101.090 ± 10.429[a]	97.056 ± 10.792[a]	100.027 ± 5.626	100.417 ± 6.093	–
Waist, cm	125.229 ± 7.953[a]	121.81 ± 7.713[a]	123.865 ± 4.216	124.817 ± 3.629	–
Hips, cm	62.686 ± 1.847[a]	60.190 ± 1.58[a]	62.440 ± 1.013	62.567 ± 0.984	–
Upper hips, cm	128.457 ± 6.206[a]	125.162 ± 6.831[a]	128.012 ± 3.843	128.794 ± 3.026	–
BMI,	40.020 ± 3.667[a]	38.441 ± 3.955[a]	39.343 ± 2.211	39.479 ± 2.054	18.5–25.0

Note: [a]significant difference ($p < 0.05$)

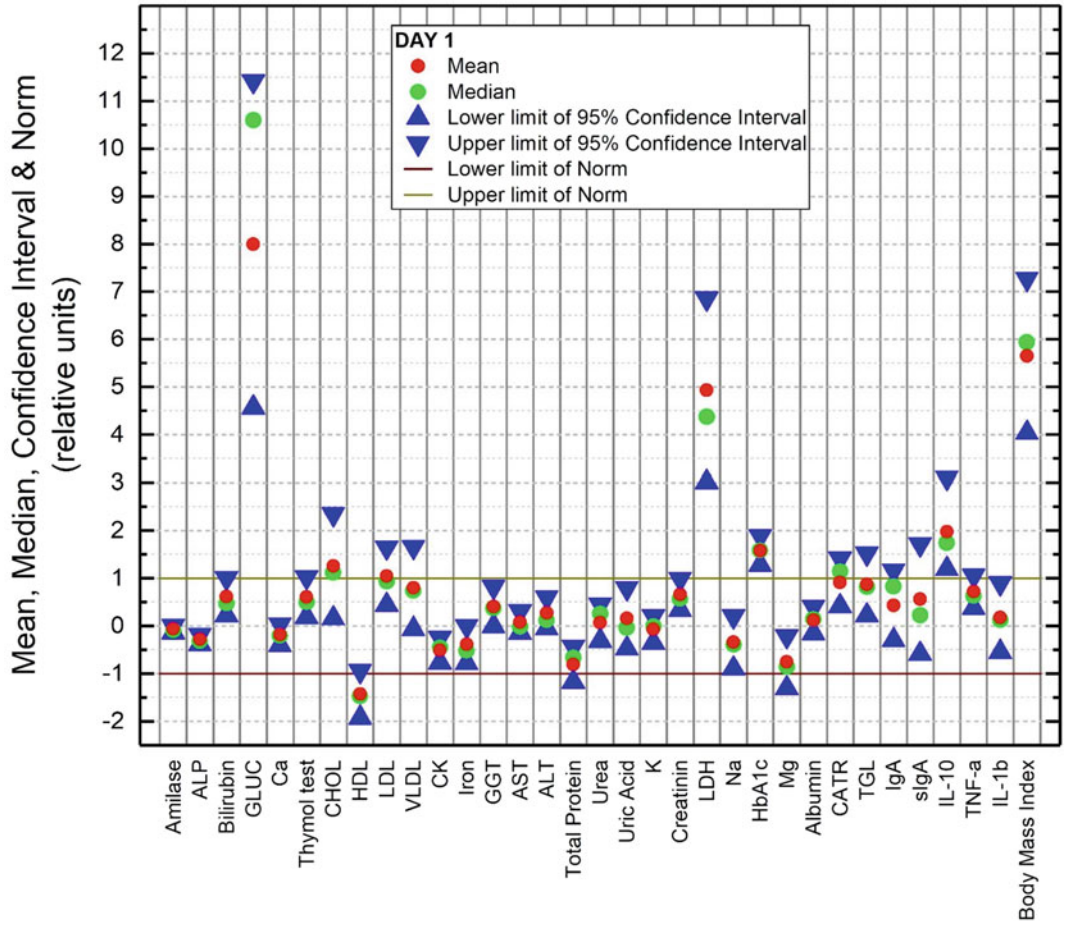

Fig. 1 Shifts for experimental group from reference ranges day 1

observation period, but a statistically significant difference appeared on day 11 compared to day 1. A statistically significant difference in the concentration of *E. coli* (lac−) was also observed on day 7 of the experiment and then an increase was observed on day 11, with a further decrease

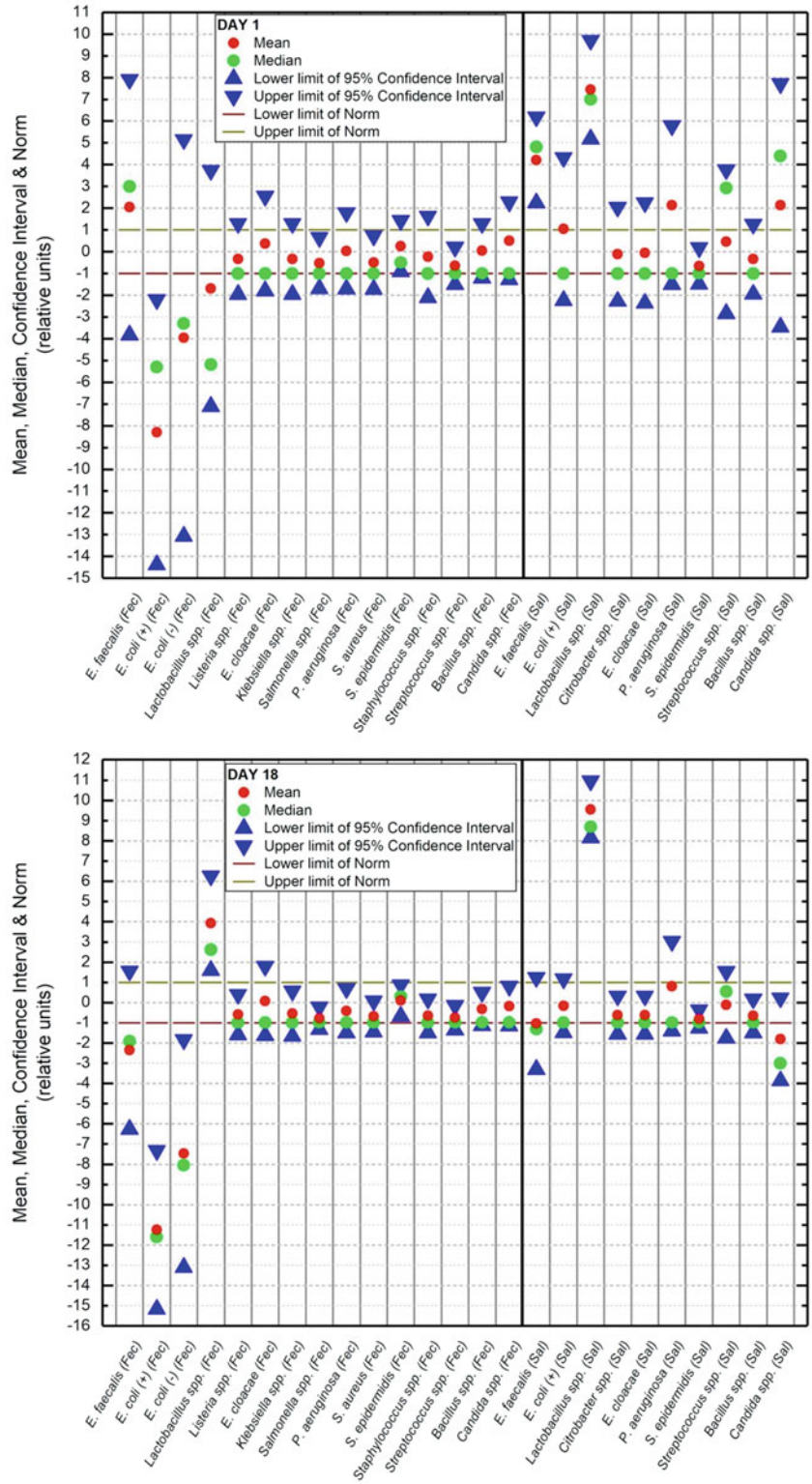

Fig. 2 Shifts of gut and oral microbiota for experimental group from reference ranges day 1 and 18

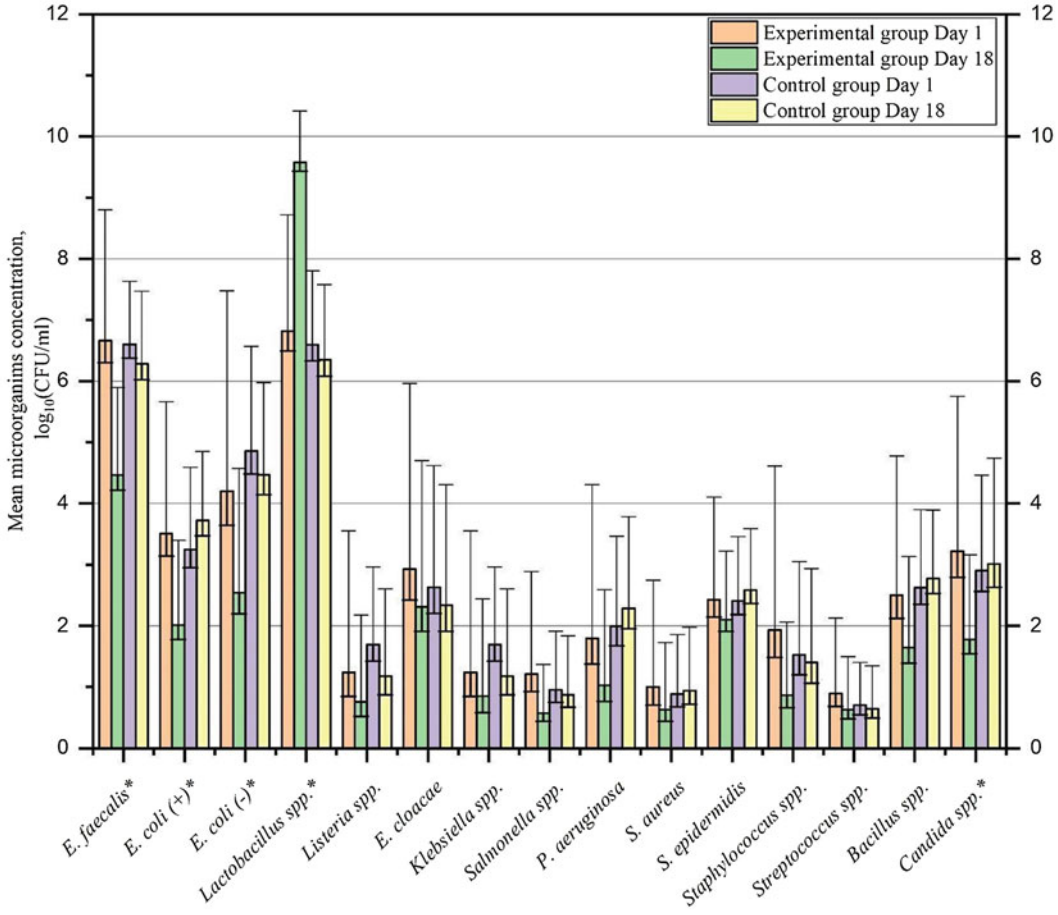

Fig. 3 Mean faecal microorganisms concentration Upper whisker –SD, lower whisker - SE
Note: * significant different values in experimental group day 1 and day 18

on day 18. The concentration of *Lactobacillus* spp. did not change statistically significantly until day 11; there was a sharp increase in concentration on day 18. For *Candida* spp., on the contrary, there was a statistically significant decrease on day 7 with the absence of statistically significant changes in subsequent days (see Fig. 5).

The oral microbiota is characterized by a sharp decrease in the concentration of *E. faecalis* on day 7 of the experiment followed by a slight decrease in concentration. *Lactobacillus* spp. demonstrated a statistically significant decrease in concentration on day 7 with further growth dynamics. The concentration of *P. aeruginosa* decreased during the experiment and a statistically significant difference was observed on day 11 compared to day 1. *Candida* spp. is characterized by a slight increase in concentration on day 7 and a further decrease until day 18 of the experiment while a statistically significant change was observed on days 7–11 and 7–18 (see Fig. 6).

4 Discussion

The issue of treatment of type 2 diabetes is still relevant. Emergence of a number of new markers greatly simplifies and increases the accuracy of the disease diagnosis, but medical personnel still mostly uses long-tested, “classic” markers of diabetes, in particular because of their availability for

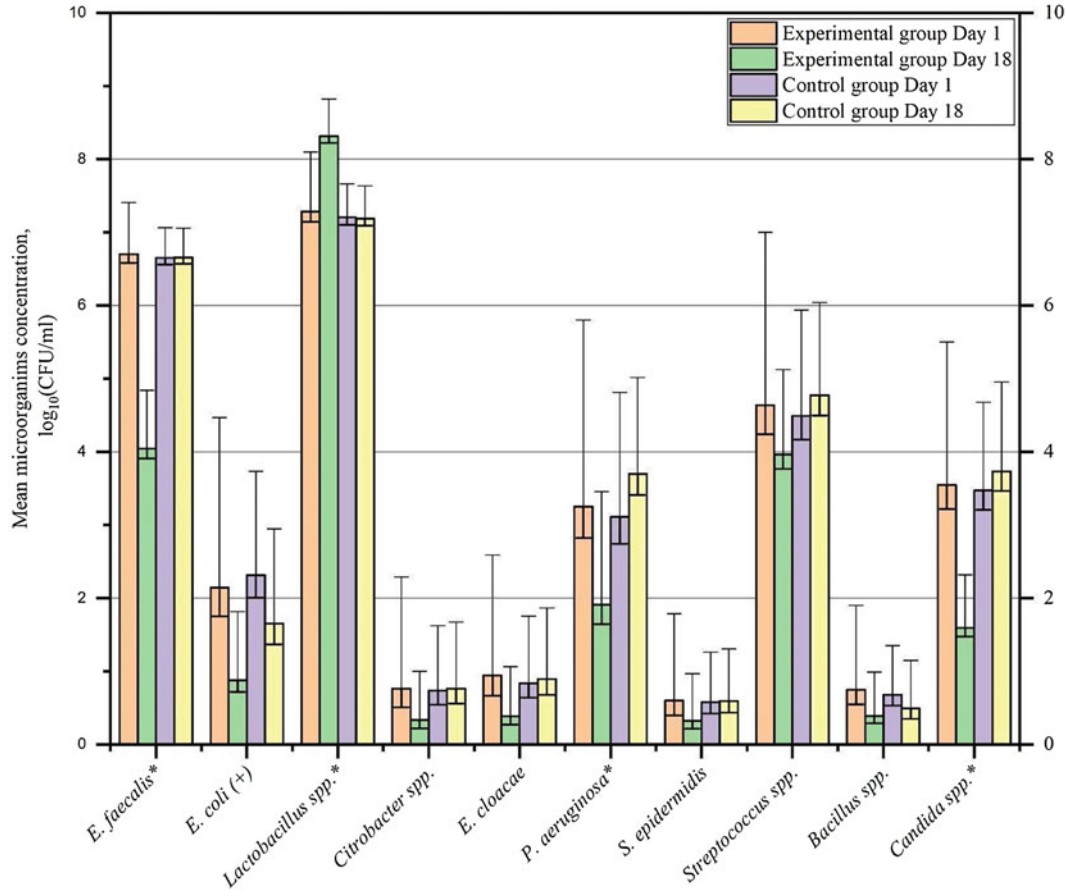

Fig. 4 Mean saliva microorganisms concentration. Upper whisker –SD, lower whisker - SE
Note: * significant different values in experimental group day 1 and day 18

analysis (DeFronzo et al. 2015). In our work, we used a classic set of such markers as well as a number of other indicators, including intestinal and oral microbiota as recent publications emphasize its significant role in the development of type 2 diabetes and human health in general (Gurung et al. 2020; Sharma and Tripathi 2019). According to the results of indicators' change during the experiment, there are improvements in a number of markers, such as VLDL, glucose, creatinine, urea, magnesium, sodium, thymol test, and uric acid.

VLDL involves pre-beta-lipoproteins that are formed in the liver and are the main transport form of endogenous triglycerides. They are classified as highly atherogenic lipoproteins involved in the formation of atherosclerotic plaques. Hance, a decrease in VLDL indicates an improvement in lipid metabolism, reducing the risk of atherosclerosis and coronary heart disease developing (Xie et al. 2017). Elevated glucose levels are one of the main diagnostic markers of T2D, and, therefore, a decrease in its level indicates that our proposed diet has a therapeutic effect.

In addition, we noticed a statistically significant decrease in creatinine and urea levels was observed in all patients in the experimental group. This change resulted from an increase in the consumption of vegetables and fruits, as well as a decrease in the consumption of meat products. According to the analysis of literature data, a decrease in the levels of these biochemical

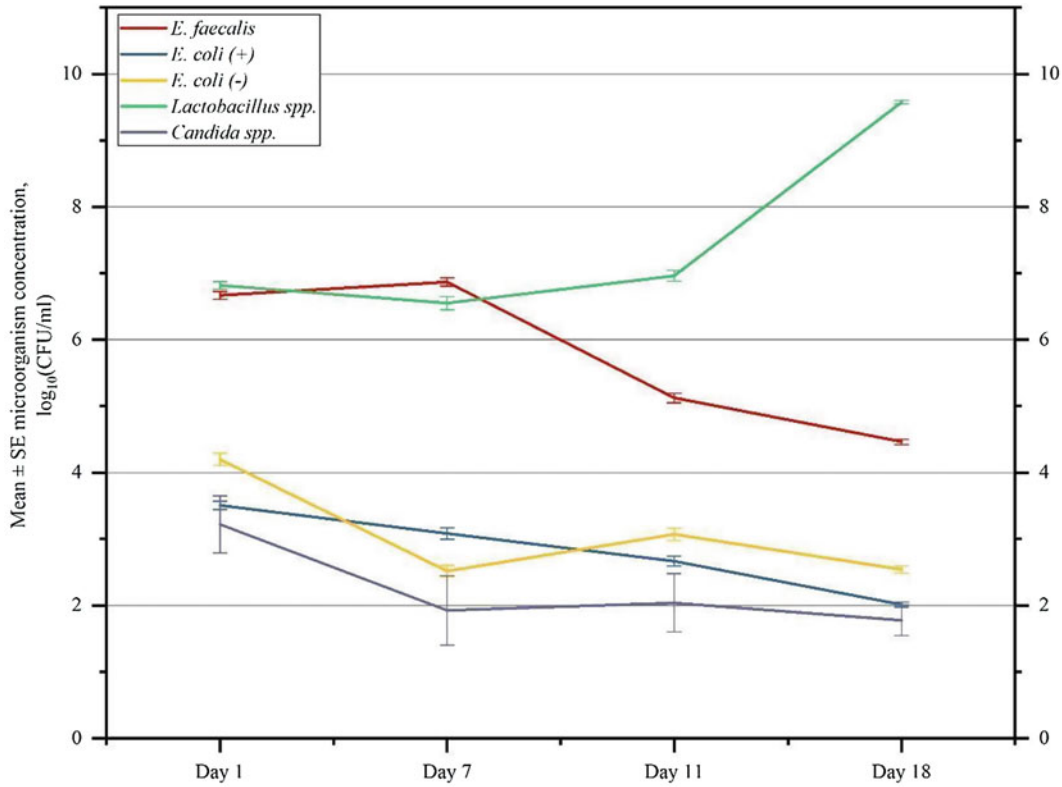

Fig. 5 Dynamic of faecal microbiota changes in experimental group. All data represented on the figure are significantly different between 1 and 18 days

parameters within normal limits may be indicative of normalization of the renal excretory function (Gowda et al. 2010).

According to previous research, diabetes mellitus is one of the diseases with increased frequency of electrolyte abnormalities given that the impaired renal function, malabsorption syndromes, and acid-base disorders are often present in diabetic patients (Liamis et al. 2014). Magnesium deficiency may relate to the development of atherosclerosis, coronary heart disease, and cardiac arrhythmias while low blood magnesium is associated with the development of insulin resistance (Kostov 2019). According to experimental data obtained, we observed an increase in the concentration of magnesium in experimental group patients compared to the control group which demonstrated a tendency to an increase in the concentration of this indicator.

It should be noted that hyponatremia is associated with increased plasma glucose concentrations (Liamis et al. 2015). As a result of adherence to the personalized diet, patients in the experimental group demonstrated an increase in the concentration of another microelement, sodium, compared to the control group, in which this indicator remained almost unchanged throughout the study. Sufficient sodium concentration is extremely important for proper functioning of membrane transport, muscle contraction, nerve impulse transmission, and many other vital functions (Constantin and Alexandru 2011), and therefore, normalization of this indicator indicates the effectiveness of the proposed diet.

A statistically significant decrease in thymol test levels within normal limits can indicate improvement of liver function (Djiambou-Nganjeu 2019). A statistically significant increase

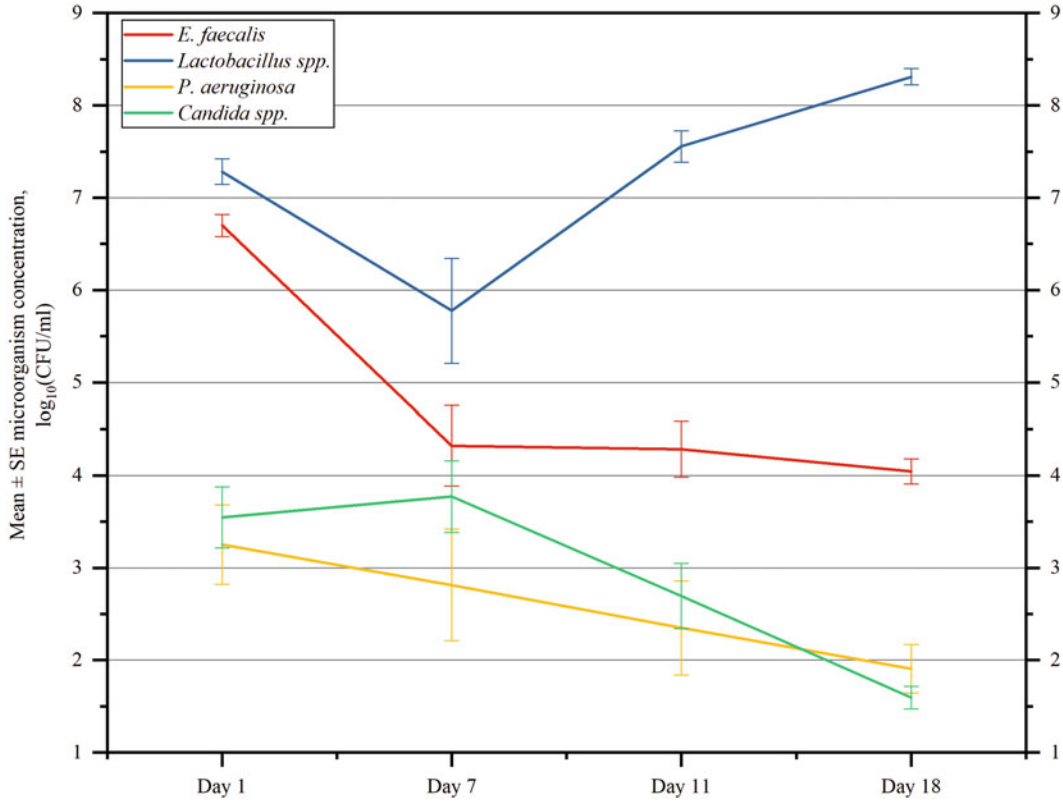

Fig. 6 Dynamic of oral microbiota changes in experimental group. All data represented on the figure are significantly different between 1 and 18 days

in uric acid levels within normal limits can be explained by the increase in the consumption of foods containing fructose, such as apples, persimmons, blueberries, pears and dried fruits.

Another important result is a change in the microbiota of the experimental group patients. The study demonstrated a statistically significant decrease in enterococci, *E. coli*, and *Candida* spp. concentration, as well as an increase in lactobacilli concentration. This indicates the normalization of intestinal microbiota, which, in turn, leads to metabolism improvement, including glucose and cholesterol metabolism (Ma et al. 2019).

All the above-mentioned changes in biochemical and immunological parameters, as well as normalization of patients' gut and oral microbiota, cause changes in patients' physical parameters, namely a statistically significant decrease in body weight and the circumference of waist, hips, and upper thighs in all patients of the experimental group.

Data on the immune status of the experimental group patients demonstrated that there is a statistically significant decrease in the levels of secretory IgA and proinflammatory cytokine TNF-α compared to the control group demonstrating a tendency to a decrease in these indicators (see Table 1). Literature analysis shows that TNF-α is considered one of the many risk factors in the development of type 2 diabetes. With regard to type 2 diabetes, it affects glucose metabolism, sensitivity of peripheral tissues to insulin, and renin-angiotensin system, and is involved in the development of oxidative stress. It possesses cytotoxic activity, promotes endothelial dysfunction, and is able to induce apoptosis of insulin producing cells (Dombrovska 2017). Thus, we

can conclude that a decrease in TNF-α level confirms the effectiveness of the proposed diet.

All this confirms the hypothesis of the possibility of personalized diet use for treatment of type 2 diabetes. In general, diets are often used in type 2 diabetes treatment. The Mediterranean diet is known to be one of the most studied diets and its positive effect on health has been proved (Trichopoulou et al. 2005). In addition, this diet is claimed to be effective in prevention and treatment of type 2 diabetes (Pérez-Jiménez et al. 2002). Research (Shai et al. 2008; Esposito et al. 2009) showed that the use of the Mediterranean diet leads to a statistically significant decrease in glucose and glycosylated haemoglobin in blood, as well as a decrease in weight and body mass index. Herewith, in our work we did not observe statistically significant changes in glycosylated haemoglobin in patients of the experimental group. This may be due to the short duration of the proposed diet (18 days), as this biochemical blood indicator reflects the average content of glucose in blood over a long period of time (3–4 months).

It should be noted that literature does not provide data on the changes in such biochemical indicators as urea, thymol test, uric acid, creatinine, sodium, and magnesium under the influence of diet in patients with diabetes mellitus. The reason may be that only classic markers of diabetes, such as glucose, glycosylated haemoglobin, cholesterol, triglycerides, low-density lipoprotein, very low-density lipoprotein, and high-density lipoprotein, are usually studied. However, most diets used in type 2 diabetes treatment do not consider the patient's condition and are not personalized. The most valuable in this regard is the known approach to adjusting body state in type 2 diabetes based on the glycaemic index (Zeevi et al. 2015). The study demonstrated the ability to predict the glycaemic response to the use of certain foods, which resulted in the possibility of making plans for personalized nutrition and adjusting intestinal microbiota. They used blood parameters, dietary habits, anthropometrics, physical activity, and gut microbiota measured to predict personalized postprandial glycaemic responses to daily consumed meals. The results of cohort study suggest that personalized diets may consistently alter gut microbiota configuration and successfully modify elevated postprandial blood glucose and its metabolic consequences. However, this approach is difficult to implement and it is not based on the use of BAS, which are extremely promising in terms of correction of not only body condition in type 2 diabetes, but also a number of other diseases.

5 Conclusions

A personalized diet based on the use of individually selected BAS and probiotic microorganisms is one of the possible ways to improve the condition of patients with type 2 diabetes. Its use in the experimental group of 35 patients led to the improvement in a number of biochemical (glucose, thymol test, VLDL, urea, uric acid, creatinine, sodium, and magnesium), immunological (sIgA, TNF-a), and all physical parameters. The intestinal and oral microbiota condition also normalized. Reduction in *E. faecalis, E. coli, P. aeruginosa,* and *Candid*a spp., as well as an increase in the number of lactobacilli, was observed. Statistically significant changes were observed in only a small number of the studied 62 markers, so it is important to identify a narrow range of priority biomarkers. The results obtained can be used for further treatment of patients with type 2 diabetes and introduction of personalized medicine in Ukraine.

Conflict of Interest The authors declare no conflicts of interest in relation to this article.

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Beyond Bone: Infectious Diseases and Immunity in Parathyroid Disorders

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Abstract

Parathyroid disorders are characterized by alterations in calcium and phosphate homeostasis due to inappropriately high or low levels of parathyroid hormone (PTH). Despite PTH receptor type 1 has been described in almost all immune lineages and calcium signalling has been confirmed as a crucial mediator for immune response, *in vitro* studies on the physiological interactions between PTH and immunity are conflicting and not representative of the clinical scenarios seen in patients with parathyroid disorders. Infectious diseases are among the main causes of increased morbidity and mortality in patients with secondary hyperparathyroidism and chronic kidney disease. More, immune alterations have been described in primary hyperparathyroidism. Recent studies have unveiled an increased risk of infections also in hypoparathyroidism, suggesting that not only calcium, but also physiological levels of PTH may be necessary for a proper immune response. Finally, calcium/phosphate imbalance could affect negatively the prognosis of infectious diseases. Our review aimed to collect available data on infectious disease prevalence in patients with parathyroid disorders and new evidence on the role of PTH and calcium in determining the increased risk of infections observed in these patients.

Keywords

Hyperparathyroidism · Hypoparathyroidism · Immune function · Infectious disease · PTH

Authors Hasenmajer Valeria and Puliani Giulia have equally contributed to this chapter.

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Abbreviations

cAMP	cyclic AMP
CKD	chronic kidney disease
PBMC	peripheral blood mononuclear cells
PHA	phytohemagglutinin

PMNL polymorphonuclear leukocytes
PTH parathyroid hormone
PTH1R type 1 PTH receptor

1 Introduction

Parathyroid disorders are characterized by alterations of parathyroid hormone (PTH) secretion, often leading to, or provoked by, an impairment in calcium and phosphate homeostasis. The hyperfunction of one or more parathyroid glands can be due to autonomous secretion of PTH (primary hyperparathyroidism) (Walker and Silverberg 2018), to a physiological response to hypocalcaemia, renal failure or vitamin D deficiency (secondary hyperparathyroidism) (Chandran and Wong 2019) or to autonomous PTH secretion after long-term secondary hyperparathyroidism (tertiary hyperparathyroidism) (Chandran and Wong 2019). Primary and tertiary hyperparathyroidism are characterized by increased calcium circulating levels and urinary excretion and decreased phosphate serum levels. Hypoparathyroidism, on the other hand, is characterized by undetectable or inappropriately low PTH secretion leading to hypocalcaemia and increased phosphate levels (Marcocci et al. 2015). Surgical lesion of one or more parathyroid gland is the main cause of hypoparathyroidism (Marcucci et al. 2018), but several other rarer aetiologies can affect PTH secretion (Mantovani and Elli 2019) and will be further discussed in the dedicated paragraph. Patients with parathyroid disorders are at an increased risk of comorbidities and concomitant diseases due to alterations of calcium homeostasis and bone and kidney disease.

Previous researches have highlighted the pivotal role of immune cells in mediating bone effects of PTH secretion. In animal models, T lymphocytes expressing CD40 ligand (CD40L) have been described as mediators of resorptive (Tawfeek et al. 2010) and anabolic (Robinson et al. 2015) effects of PTH. In fact, deletion of T Lymphocytes expressing CD40L, the PTH receptor (Tawfeek et al. 2010) or an impairment of antigen presentation (Bedi et al. 2010) blunted osteoclastogenic activity in experimental models of hyperparathyroidism (Gao et al. 2008). On the other side, T Lymphocytes also mediate the anabolic effects of intermittent PTH therapy (Terauchi et al. 2009), activating a sclerostin-independent pathway (Li et al. 2014). Regulatory T Lymphocytes are also expanded by intermittent PTH therapy (Yu et al. 2018), while Interleukin 17 producing T Lymphocytes increase its catabolic effects (Pacifici 2016).

More recently, a significant increase in infectious diseases and immune alterations in hyper- and hypoparathyroidism suggested a possible role for PTH-immune interactions beyond the osteoimmune niche in maintaining immune homeostasis and mediating systemic effects of alterations in PTH secretion.

PTH action is mediated by its receptors, PTHR1 and PTHR2. Both are group B canonical G protein-coupled receptors. PTHR1 binds both PTH and PTH related peptide (PTHrP), while PTHR2 is selective for PTH. For the scope of this review, we will focus on PTHR1 function given its role in calcium and bone homeostasis and the lack of data on PTHR2 and immunity. Conformational change of PTHR after ligand binding activates several intracellular pathways including primarily the Gs, cyclic AMP (cAMP) protein kinase A (PKA) cascade and the Gq, phospholipase C (PLC), Ca^{2+} and protein kinase C (PKC) signalling, even though activation of phospholipase D and mitogen-activated protein kinase (MAPK) signalling cascades have been described (Urena et al. 1993). While most studies have focused on the cAMP signalling pathway that mediates the osteoanabolic effects of PTHR1 binding, downstream signalling of PTHR1 activation directly modulates immune function, for example by increasing TNF secretion in T Cells (Tawfeek et al. 2010) or modulating the nuclear factor of activated T-cells (NFAT) family (Huang et al. 2010). Furthermore, PTH and Ca^{++} signalling are significantly intertwined.

Calcium levels are one of the main regulators of the immune response, due to the role of ionized calcium in intracellular signalling. Intracellular

calcium levels are tightly regulated by a variety of mechanisms, in which parathyroid hormone (PTH) plays a significant role. As extensively demonstrated in adrenal disorders (Isidori et al. 2018; Bancos et al. 2017) the immune system appears tightly correlated with the endocrine function as both react in a coordinated manner to face external challenges. The presence of the PTH receptor on immune cells suggests a physiological role in the immune response that extends beyond the regulation of bone remodelling.

Despite the increasing body of evidence on immune derangement in parathyroid disorders, results are often contradictory and data on the direct effect of PTH on immune homeostasis is scarce. In this context, the aim of this review was to summarise available evidence on prevalence, severity, predisposing factors and underlying mechanisms of infectious diseases in parathyroid disorders; to highlight contradictory conclusions and confounding factors possibly affecting the obtained results; and to provide more solid basis for further research on immunity in hyper and hypoparathyroidism.

2 Methods

We performed a research of published literature with no time constraints using the following keywords: "PTH", "parathyroid hormone", "hypoparathyroidism", "hyperparathyroidism", "primary hyperparathyroidism", "secondary hyperparathyroidism", "parathyroid disorders", "immune system", "immunity", "autoimmune diseases", "infectious diseases", "infections". Only English papers were included. Being out of the main topic of this review, papers on infections as a side effect of thyroid or parathyroid surgery were excluded, along with papers focusing on the aetiology and physiopathology of autoimmune hypoparathyroidism. The review has been conducted according to SANRA scale for the quality assessment of narrative review articles (Baethge et al. 2019).

3 PTH in the Healthy Immune System

Studies on the interactions between calcium homeostasis, PTH and the immune system were prompted in the late 1970s by the discovery of the players, subsequently identified as immune cells, mediating the interaction between PTH, bone resorption and osteoclasts, and the identification of PTH receptor on their surface (Perry et al. 1984; Yamamoto et al. 1983; Stock and Coderre 1982; Minkin et al. 1977). Early studies evaluated alterations of cyclic AMP (cAMP) concentrations or radionuclide binding as indirect indicators of PTH1R expression and activity. PTH1R binding in vitro targeted mononuclear leukocytes (Perry et al. 1984; Yamamoto et al. 1983; Stock and Coderre 1982; Minkin et al. 1977), while polymorphonucleates and red blood cells did not seem to express PTH1R (Yamamoto et al. 1983). Narrowing the analysis, studies on cAMP accumulation showed direct effects of PTH on monocytes, but not on lymphocytes (Stock and Coderre 1982), even though these results were conflicting with others identifying lymphocytes as a major target of PTH (Perry et al. 1984; Yamamoto et al. 1983). These contradictory findings are probably due to heterogeneity of methods used such as different concentrations of rat or bovine PTH, different duration of incubation, sampling lymphocytes from healthy donors or uremic patients or evaluating PTH binding via radionuclide analysis or other parameters such as rosette formation.

Functional studies on the effects of PTH on immune cells also showed conflicting results. A stimulatory effect was observed in mast cells, with increased release of mediators after antigen stimulation in cells pre-treated with 1-34 PTH (Simpson et al. 1991). 1-84 and 1-34 PTH stimulated T lymphocyte proliferation in vitro, with no effects on CD4/CD8 ratio, and increased cAMP production (Klinger et al. 1990). The authors speculated that, *in vitro*, acute administration of PTH elicits a different response when

compared to prolonged exposure to steadily high PTH, mimicking hyperparathyroidism (Klinger et al. 1990). In fact, *in vitro* studies with supra-physiological concentrations of PTH have shown a dose-dependent suppressive effect on lymphocytic function, and a decreased helper/suppressor ratio (Shasha et al. 1988). More recent studies have mostly focused on the interactions between PTH and immune cells in the osteoimmune niche, and an exhaustive description of these results is out of the scope of this review.

While the results on the *in vitro* effects of PTH on immune cells are insightful because free of clinical confounding factors, these experiments are far from resembling the complex scenarios of human parathyroid disorders, where an impairment in the immune system is now becoming a clearer data (Underbjerg et al. 2018).

4 Infectious Diseases and Immune Alterations in *Secondary Hyperparathyroidism* due to Chronic Kidney Failure

Secondary hyperparathyroidism is a frequent complication of chronic kidney disease (CKD), as a response to renal failure and the consequent imbalance in electrolyte excretion and resorption. In particular, hyperphosphatemia, due to impaired phosphate excretion, is the main driver of hyperparathyroidism in CDK, followed by the reduction of serum ionized calcium (Chandran and Wong 2019). Long-standing secondary hyperparathyroidism can lead to tertiary hyperparathyroidism, characterized by the autonomous activity of parathyroid gland(s) that cause hypercalcemia (Chandran and Wong 2019).

High levels of PTH and CKD are tightly intertwined, and many efforts have been paid during the last decades to unveil the possible relationship between hyperparathyroidism and the complications that characterized early and late stages of renal failure. Infectious diseases, together with cardiovascular diseases, are the main causes of morbidity and mortality in patients affected by CKD (Goldblum and Reed 1980). The annual mortality for sepsis is up to 300-fold higher in dialysis patients compared to the general population (Sarnak and Jaber 2000; Powe et al. 1999), and patients also showed poor vaccination responses (Girndt et al. 1995) and high incidence of infections (Goldblum and Reed 1980).

The risk of infections is higher also in earlier stages of CKD. In an observational cohort study on 9697 participants (aged 53–75 years), the decrease in glomerular filtration rate was associated with a progressive increase in the hazard ratio for both infections and infection-related deaths (Ishigami et al. 2017). Similar findings have been reported also for community-acquired infections, with an incidence of 74/1000 person-years in case of normal eGFR and 419/1000 person-years in case of glomerular filtration rate lower than 30 ml/min (Xu et al. 2017).

The underlying immune function impairment is caused by many different mechanisms as uremic toxins (including cytokines accumulation), pro-inflammatory status, complement activation, chronic inflammation, malnutrition (Syed-Ahmed and Narayanan 2019) and it is not possible to isolate the contribution of every single factor. Platinga et al., in a study on 1010 dialysis patients in the USA, demonstrated a higher incidence rate for all infections in patients with high phosphate levels at baseline, early after the start of dialysis, when compared to patients with normal phosphate levels, even after adjustment for PTH levels, dialysis dosage, and vitamin D supplements (Plantinga et al. 2008). In this study, increased phosphate seems an independent risk factor for infections. Considering infection type, sepsis and osteomyelitis were more frequent than in the case of normal phosphate levels. In CKD, the levels of FGF23, a hormone responsible for renal excretion of phosphate and vitamin D metabolism, are higher from the early phase of the disease (Wahl and Wolf 2012) and are associated to increase mortality. Recent evidence supports the detrimental role of FGF23 in innate immune function, and in particular in macrophage, blocking M2 polarization and reducing

recruitment of polymorphonuclear leukocytes (PMNL) (Fitzpatrick et al. 2018) and increasing pro-inflammatory chemokines (Wallquist et al. 2018). FGF23 acts also indirectly through a reduction in active vitamin D levels. Even if high phosphate levels are the main stimulator of FGF23 synthesis, also PTH is able to induce FGF23 transcription in bone cells (Lanske and Razzaque 2014).

In addition to the abovementioned factors, also chronic secondary hyperparathyroidism can play a role in immune dysregulation since high PTH levels have been associated with an impaired innate and acquired immune response (summarized in Table 1).

High levels of PTH in dialysis patients cause impaired phagocytosis of PMNL, through decreased ATP content, elevated basal levels of cytosolic calcium, and, consequently, a smaller rise in intracellular ionized calcium levels in response to antibody (Alexiewicz et al. 1991). Moreover, prolonged exposure to PTH significantly inhibited the random migration of PMNL (Doherty et al. 1988) and impaired the bactericidal activity of PMNL mediated by the generation of oxidizing radicals (Kiersztejn et al. 1992). To confirm that high PTH levels, and not only renal failure, were the cause of impaired phagocytosis, Chervu et al. compared rats affected by chronic renal failure subjected or not to parathyroidectomy or verapamil treatment. The study demonstrated that phagocytosis was impaired in animals with high PTH and intracellular calcium levels, which determine a reduction in ATP levels and an increase in cytosolic basal calcium levels, according to earlier observations. To investigate whether PTH binding or PTH-mediated increase in basal intracellular calcium levels caused the alterations in phagocytosis, the authors showed that parathyroidectomy and verapamil treatment restored normal phagocytosis (Chervu et al. 1992), demonstrating the interdependence of hyperparathyroidism and alterations of intracellular calcium balance. Studies on human subjects demonstrated the role of calcium antagonists in improving PMNL phagocytosis in the uremic state as well (Massry and Smogorzewski 2001). An interventional trial on CKD patients demonstrated that parathyroidectomy (and consequent normalization of PTH levels) can reduce, but not normalize, intracellular calcium levels in PMNL (Deicher et al. 2005).

Overall, animal studies seem to confirm the direct role of high PTH in causing PMNL impairment, while human studies suggest that high PTH is probably involved in altered PMNL phagocytosis even if it is not the only factor, as confirmed by the partial positive effect of parathyroidectomy.

Not only innate but also acquired immunity may be altered in secondary hyperparathyroidism due to CKD. In dialysis patients, T cell function is impaired. T cells are less responsive to IL-2 and mitogens stimulation (Alexiewicz et al. 1990a), and serum from uremic patients inhibited T cell response *in vitro*, but this inhibition decreased significantly when samples were collected from the same patients after parathyroidectomy (Giacchino et al. 1985), suggesting a direct contribution of high PTH levels on altered T cell response. As observed in PMNL, the main reason for the detrimental action of PTH on T cells is the high resting levels of intracellular ionized calcium (Alexiewicz et al. 1990a; Ori et al. 1999), which alter calcium homeostasis and dampen intracellular calcium variations. Calcium signalling is essential for cell proliferation and cytokine activation, through the action of the calcium-dependent ATPase (Ori et al. 1999). In addition, the total number of T lymphocytes is reduced in haemodialysis patients, and these results have been indirectly associated with osteoprotegerin, which is increased in renal failure and may contribute to immune response downregulation (Eleftheriadis et al. 2013).

Subset redistribution has also been described in patients with secondary hyperparathyroidism. Considering T cells subpopulation analysed by flow cytometry in haemodialysis patients with normal (16 patients) or high (18 patients) PTH levels, Griveas et al. demonstrated an increase of CD2, CD3, CD3/CD8, CD3/CD4 and CD4/CD8 ratio in the group with high PTH (Griveas et al. 2005). Conversely, Angelini et al. demonstrated a decrease in CD4 subpopulation and an increase in CD8 subpopulation, that only occurred in patients

Table 1 Effects of high levels of PTH on immune cells in secondary hyperparathyroidism due to chronic kidney disease (CDK)

Main PTH-related immune function alterations in CKD patients		
Cells	Alterations	References
T cells	Reduced response to mitogens and IL-2 Reduced proliferation Reduced T cells count Decrease of CD4/increase of CD8 Th17 cells are increased whereas Treg cells are decreased	Alexiewicz et al. (1990a) Ori et al. (1999) Eleftheriadis et al. (2013) Ozdemir et al. (2002), Angelini et al. (1993) Lang et al. (2014)
PMN	Impaired phagocytosis Inhibited the random migration Impaired the bactericidal activity	Alexiewicz et al. (1991), Chervu et al. (1992) Doherty et al. (1988) Kiersztejn et al. (1992)
B lymphocytes	Reduced response to mitogens Reduced proliferation Reduced immunoglobulin production	Alexiewicz et al. (1990b), Gaciong et al. (1991) Raskova et al. (1987), Smogorzewski and Massry (2001) Raskova et al. (1987), Gaciong et al. (1991)

with hyperparathyroidism and not in patients with normal PTH levels (Angelini et al. 1993). A reduction of $CD4^+/CD8^+$ lymphocyte ratio in patients with hyperparathyroidism was also confirmed by Ozdemir et al. (Ozdemir et al. 2002). In haemodialysis patients Th17 cells are increased whereas Treg cells are decreased, with, respectively, a positive and negative correlation with phosphate levels (Lang et al. 2014). These findings could be important in clinical practice since Th17 cells are involved in the pathogenesis of autoimmune disease while Treg reduces T cell response; an imbalance between Th17 and Treg function could facilitate the inflammatory process typical of CKD (Lang et al. 2014).

Not all studies demonstrated a reduction in T cell response in CKD patients, but the heterogeneity of results could be due to different stimuli utilized in experimental settings. A study using not phytohemagglutinin (PHA), which can stimulate T response through T cell receptor, but staphylococcal enterotoxin B, which links also class 2 MHC complex, failed to demonstrate a difference between haemodialysis patients with high o normal PTH levels (Eleftheriadis et al. 2007). Furthermore, results from clinical studies on CKD patients could provide different results compared to in vitro analyses of PTH effects on lymphocytes from healthy donors. In fact, this possible discrepancy with the T cells alteration observed in CKD could be explained by the prolonged exposure to PTH which is typical of dialysis patients (Klinger et al. 1990; Shurtz-Swirski et al. 1995).

The function of B lymphocytes is also impaired in CKD. The proliferation of B cells, both dependent and independent from T-cells, is reduced in patients affected by CKD (Raskova et al. 1987). This reduction cannot be completely attributed to PTH excess even if it has been demonstrated that PTH is able to reduce significantly B cell proliferation (Smogorzewski and Massry 2001). Moreover, functional studies have shown that PTH can reduce B cell proliferation and function through an increase in cAMP (Alexiewicz et al. 1990b). As in T cells, the resting levels of intracellular ionized calcium are higher in B cells in CKD patients (Smogorzewski and Massry 2001) and nifedipine is able to restore intracellular calcium levels in B cells (Alexiewicz et al. 1997). Different studies demonstrated a reduction in immunoglobulin production in CKD patients in response to mitogen (Raskova et al. 1987) or various vaccines including hepatitis B (Kohler et al. 1984) and influenza (Cappel et al. 1983). Immunoglobulin production in response to Staphylococcus aureus Cowan I or with pokeweed mitogen was also lower in haemodialysis patients than controls and the treatment with PTH, at the initiation of B-cells culture, reduced IgG, IgM and IgA production by B lymphocytes (Gaciong et al. 1991).

Tsanno-Martins et al. analysed the effect of parathyroidectomy on T- and B-cell function of haemodialysis patients affected by severe secondary hyperparathyroidism, demonstrating an increase of the lymphoproliferative response to PHA and pokeweed mitogen after surgery (Tzanno-Martins et al. 2000), suggesting a direct role of lowering of PTH levels in obtaining normal T- and B-cell function.

A possible role for vitamin D has also been proposed in modulating immune function in patients with secondary hyperparathyroidism. Martinez et al. evaluated the presence of 1,25OH vitamin D receptor in PBMC: compared to healthy controls, the number and the maximal binding capacity of the receptors was lower in patients affected by secondary hyperparathyroidism and increased in case of primary hyperparathyroidism. After parathyroidectomy for primary hyperparathyroidism or kidney transplant for secondary hyperparathyroidism, the number of receptors normalized (Martinez et al. 1994).

In conclusion, even if the role of uremic toxins, malnutrition, vitamin deficiency, and drug therapy cannot be ignored (Smogorzewski and Massry 2001), the secondary hyperparathyroidism contributes to the immune system derangement in patients with CKD, and clinicians should, therefore, pay special attention to prophylaxis, patient education and prompt therapeutic measures due to the high risk of infections in these patients.

5 Infectious Diseases and Immune Alterations in *Primary Hyperparathyroidism*

Primary hyperparathyroidism is a common endocrine disease, characterized by hypercalcemia and elevated or inappropriately normal levels of parathyroid hormone (Walker and Silverberg 2018). In most cases, it is caused by a solitary parathyroid adenoma, while diffuse hyperplasia, multiple adenoma, and parathyroid carcinoma are less frequent (Marcocci and Cetani 2011). The classical clinical presentation of primary hyperparathyroidism includes skeletal, renal, gastrointestinal, neurological, and psychiatric manifestations (Cope 1966), even if more than 80% of patients in Europe and the USA are diagnosed incidentally, during biochemical examinations (Walker and Silverberg 2018).

An increased risk of infections has been described in patients affected by primary hyperparathyroidism and this disease could be used as a model for evaluating the role of PTH itself in immune function impairment because it is not burdened by the other systemic alterations that characterize CKD (Shasha et al. 1989). Nevertheless, only a few studies have focused on immune function in primary hyperparathyroidism.

In a study on 3 patients and 3 controls, Shasha et al. demonstrated a reduction in T cells count in patients (−40%), with an impaired response to the stimulation with PHA, that was restored after parathyroidectomy (Shasha et al. 1989). Patients also showed a decrease in $CD4^+$ and an increase in $CD8^+$ lymphocytes, while after surgery CD4/CD8 ratio increased (Shasha et al. 1989). A subsequent study on 12 patients and 9 controls confirmed that response to PHA was reduced in patients and that the observed alterations were restored after parathyroidectomy; however, this study did not confirm a reduction in peripheral blood mononuclear cells (PBMC) subpopulations in patients compared to controls, except for an increase in CD4 and a decrease in CD8 before parathyroidectomy, also associated with a reduced response to activation markers as IL-2 and transferrin receptor (Kotzmann et al. 1998). In primary hyperparathyroidism, neutrophil leukocyte chemotaxis was altered compared to the control group and was restored after parathyroidectomy (Nordenstrom et al. 1989). Differently from secondary hyperparathyroidism, Elias et al. demonstrated that serum from patients affected by hyperparathyroidism was able to promote both patients' and controls' lymphocyte proliferation after stimulations with mitogen (Elias et al. 1982); these findings are consistent with the concept that not only PTH *per se* but also the length of exposure plays a role in immune function derangement. All in all, while studies are concordant in describing a decreased response to mitotic stimuli from adaptive immunity,

published literature does not provide a consistent immune profiling or model for immune derangement under continuously increased PTH secretion, and the small size of available studies do not support causal speculation on the underlying mechanisms.

On the other hand, hyperparathyroidism has been associated also with autoimmune and haematological diseases related to B cell hyperactivations, as reported in case reports, in which parathyroidectomy reversed the condition (summarized in (Canas et al. 2013)). The authors hypothesized that PTH may stimulate B lymphocyte activity and differentiation into plasma cells with the subsequent non-physiological production of antibodies (Canas et al. 2013). Moreover, hyperparathyroidism is associated with low chronic inflammation, and this could also explain the increased cardiovascular risk of these patients (Christensen et al. 2012). This twofold effect on the immune system is common to many other immune-modulatory hormones. Hypercortisolism is an excellent paradigm of this concept: while at therapeutic dosages glucocorticoids usually act as powerful anti-inflammatory agents, chronic exposure to supra-physiological and anti-circadian cortisol levels can lead to low-grade inflammation, increased metabolic and cardiovascular risk (Sbardella et al. 2018) and immune derangement (Hasenmajer et al. 2020).

In conclusion, primary hyperparathyroidism seems associated to immune system rearrangement, with on one hand an increased risk of infections, which seems to be reduced by parathyroidectomy, and on the other hand, an increase in low chronic inflammation that, in some cases, can lead to the development of autoimmune and haematological diseases.

6 Infectious Diseases in *Hypoparathyroidism*

Hypoparathyroidism is a chronic and relatively rare disease characterized by inappropriately low levels of PTH. Hypoparathyroidism is characterized by impaired calcium resorption and reduced phosphate excretion leading to hypocalcaemia and hyperphosphoremia (Mannstadt et al. 2017). To maintain physiological levels of circulating calcium, patients with hypoparathyroidism require replacement therapy with active vitamin D metabolites and calcium supplements. More recently, synthetic and recombinant PTH replacement has been proposed for hypoparathyroidism, allowing patients to lower and even discontinue the "conventional" regimen (Mannstadt et al. 2013).

However, most studies on hypoparathyroidism have been focusing on its aetiology and biochemical control of the disease, while data on comorbidities, mortality, and concomitant diseases were scanty or from preclinical studies. To investigate these overlooked aspects, retrospective studies on nation-wide cohorts have been recently conducted, unveiling some unexpected results.

Due to previous studies reporting a protective effect of vitamin D against infections (Holick 2007), in a retrospective study on a Danish Nationwide cohort the authors investigated the risk of infections in patients with hypoparathyroidism, showing an unexpected increased risk of infectious diseases (Underbjerg et al. 2014).

Since hypoparathyroidism can be due or associated with autoimmune diseases or conditions characterized by an impaired immune response such as Di George syndrome, to avoid confounding factors, only patients with post-surgical hypoparathyroidism due to non-malignant causes were included in the study.

Patients with hypoparathyroidism showed an increased risk of hospitalization due to infectious diseases, with a Hazard Ratio of 1.42. Since patients with hypoparathyroidism are at increased risk of renal disease, nephrocalcinosis, and hypercalciuria due to the impairment of calcium resorption in the renal tubule, the authors also performed a risk evaluation excluding urinary tract infections, with a persistently increased overall risk. Furthermore, to rule out opportunistic or health-care-related infections, episodes within 90 months from hospital discharge for any cause were excluded, without altering the

previously observed increased risk. Interestingly, patients with hypoparathyroidism also had shown a tendency towards a lower risk for malignancies, even though the results did not achieve statistical significance (Underbjerg et al. 2014).

Undjerberg et al. also evaluated patients with non-surgical hypoparathyroidism from the Danish registries (Underbjerg et al. 2015). According to the results from the post-surgical population, patients with non-surgical hypoparathyroidism were at an increased risk for hospitalization due to infectious diseases as well, with a higher prevalence of upper airway and urinary tract infections compared to controls. The total number of infections, with or without including urinary tract infections, was also increased (Underbjerg et al. 2015). As for malignancies, patients with non-surgical hypoparathyroidism showed a significantly lower risk of developing any malignancy and gastrointestinal cancer (Underbjerg et al. 2015).

To correlate the observed increased risk for long-term comorbidities with biochemical parameters mirroring disease control and therapy efficacy, data from all patients with hypoparathyroidism were included in a case-control study estimating the risk of complication according to biochemical findings (Underbjerg et al. 2018). For what concerns infectious diseases, results from the study showed a significant correlation between infections and higher time-weighted average serum phosphate concentration, along with hypercalcaemic episodes. High plasma phosphate and higher calcium-phosphate product were also associated with mortality in hypoparathyroidism patients, highlighting the importance of phosphate homeostasis in hypoparathyroidism (Underbjerg et al. 2018). Increased phosphate levels are also present in secondary hyperparathyroidism associated to CKD, however data on immune effects of phosphate are lacking. Examining only the hypoparathyroidism cohort, patients assuming relatively high doses of activated vitamin D showed a reduced number of infectious diseases compared to their counterparts (Underbjerg et al. 2018), suggesting a protective role for higher vitamin D dosages against microbial agents. However, most patients with secondary hyperparathyroidism assume relatively high doses of active vitamin D metabolites and infection rate and severity is significantly increased in this population, suggesting that the protective action of vitamin D can only slightly reduce the infective risk when other factors such as electrolyte imbalance or severe alterations of PTH secretion are involved.

Data on the higher prevalence of infections in hypoparathyroidism have been recently confirmed from a study on a Scottish cohort (Vadiveloo et al. 2019), showing an increased adjusted Hazard Ratio for infectious diseases in hypoparathyroidism compared to controls. When stratification for aetiologies was performed, though, the risk was increased only in the hypoparathyroidism due to non-surgical causes and hypomagnesemia groups (Vadiveloo et al. 2019). According to the Danish cohort, mean serum calcium concentration did not associate with infections, while a significant association with mortality and renal failure was unveiled (Vadiveloo et al. 2019).

A very recent study (Puliani et al. 2021) has analysed immune profiling in patients with chronic post-surgical hypoparathyroidism under conventional replacement compared to matched healthy controls. Study results have shown significant alterations in hypoparathyroidism patients with reduced circulating monocytes (nearly halved compared to controls). Patients also showed decreased $CD4^+$ T lymphocytes, $CD4^+$ T regulatory lymphocytes and $CD4^+$ Naïve T Lymphocytes compared to controls and increased NK cells. All the altered immune parameters were directly correlated with ionized and total calcium and PTH levels and inversely correlated with phosphate levels, while only NK cells number was directly correlated with Vitamin D levels. Moreover, patients showed decreased expression of inflammatory cytokines such as TNF and granulocyte monocytes colony stimulating factor (GM-CSF). In this study, patients with hypoparathyroidism reported increased number of urinary

tract infections and longer duration of respiratory tract infections compared to controls. The authors also evaluated PTHr1 expression in patients and controls, confirming the presence of PTHr1 on all immune lineages in peripheral blood mononucleated cells. The number of cells expressing PTHr1 was lower in hypoparathyroidism, but the intensity of expression was increased. Immunofluorescence analysis showed surface and cytosol binding for PTHr1 (Puliani et al. 2021).

In conclusion, data from retrospective studies have recently demonstrated an increase in infectious diseases, pointing towards a physiological role of adequate PTH levels in maintaining a healthy immune response. However, in these epidemiological studies, analysis of biochemical parameters only showed a significant correlation with phosphate and calcium-phosphate product, while no correlations with calcium levels or other parameters were unveiled. Insights from a recent study demonstrated significant alterations in immune profiling in patients affected by hypoparathyroidism under conventional replacement therapy, suggesting possible underlying mechanisms for the observed increase in infectious diseases but further confirmation studies are needed (immune alterations in hypo- and hyperparathyroidism are summarized in Fig. 1).

7 PTH Disorders in the Context of Other Syndromes

PTH alterations may be isolated or part of other genetic syndromes or autoimmune poliendocrinopathy. 22q11.2 deletion syndrome (22q11.2DS) is a complex of immune imbalance, endocrine disease, congenital heart disease, palatal abnormalities, cognitive deficits and neuropsychiatric illnesses and immune alteration due to 22q11.2 microdeletion (McDonald-McGinn et al. 2015). Actually, the term Di George Syndrome is reserved to patients with the same clinical presentation, without harbouring 22q11.2 deletion (McDonald-McGinn et al. 2015). 22q11.2DS occurs in approximately 1:4000 births (McDonald-McGinn and Sullivan 2011). During childhood, immunodeficiency present in about 75% of patients, and it is caused by thymic hypoplasia and impaired T cell production. Patients show a reduced number of CD3+ T cells and IgA deficiency can also be present (Smith et al. 1998). The infectious rate of affected patients is significantly higher, and recurrent sinusitis or otitis media involve at least one third of patients; recurrent lower airway infections are also increased (Jawad AF, 11713452) while opportunistic infections are rare (Ryan et al. 1997). On the other hand, allergic disease (Staple et al. 2005) and all kind of autoimmune disorders are common in these patients. The latter present in about 10% of patients (McDonald-McGinn and Sullivan 2011). In case of complete thymic aplasia, T cells can be absent. Clearly, these patients can necessitate thymus transplant or a fully matched T-cell transplant (McDonald-McGinn and Sullivan 2011). No data on the possible collaboration of hypoparathyroidism in causing immune derangement in 22q11.2 DS/Di George syndrome are available, although pathogenesis of these alterations has been described and does not involve PTH imbalances.

Hypoparathyroidism can also be part of Autoimmune Polyglandular Syndrome type 1 (APS-1), which is characterized by the concomitant presence of at least two of three of the following chronic mucocutaneous candidiasis, hypoparathyroidism and adrenal insufficiency (Guo et al. 2018). Other autoimmune diseases may be present, such as type 1 diabetes mellitus, autoimmune hepatitis, hypothyroidism and hypergonadotropic hypogonadism (Perheentupa 2006). The syndrome is caused by a mutation in autoimmune regulator (AIRE) gene (Rizzi et al. 2006), which encodes for a transcription factor implicated central tolerance. In fact, AIRE protein promotes negative selection of T cells, particularly in the thymus (Anderson and Su 2016). The impact of APS-1 on immune regulation is dual: on one hand, affected patients develop autoantibodies against tissue-specific antigens; on the other hand, patients can present candidiasis and other infections of oral mucosa, nails and esophagus, which usually required specific treatment and can lead to severe complication (Guo et al. 2018;

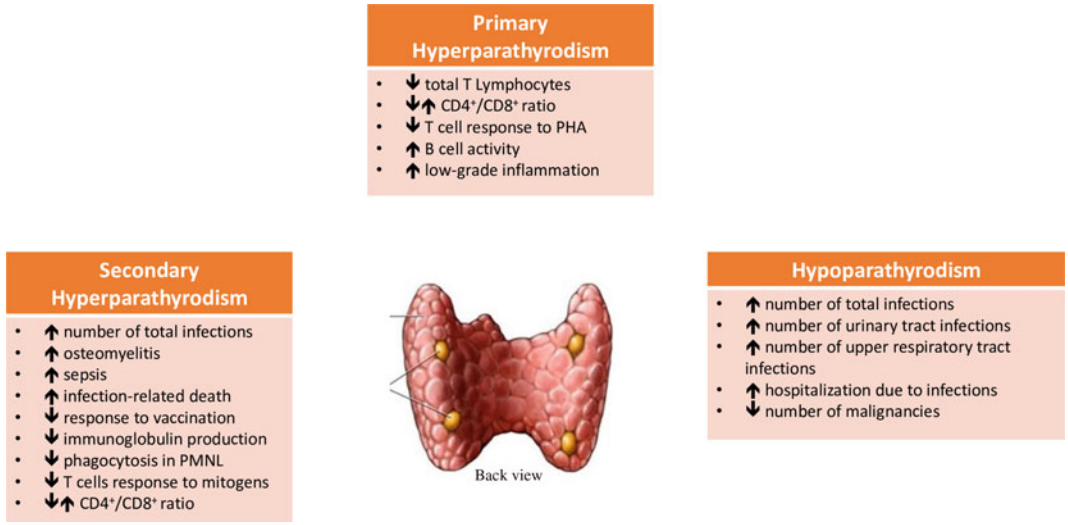

Fig. 1 Infection and immune-related complications in parathyroid disorders

Matheson and Mazza 2017). Potential contribution of hypoparathyroidism in candidiasis and infections in APS-1 patients is unknown.

Severe infections have been linked also to Hypoparathyroidism-Retardation-Dysmorphism (HRD) Syndrome. This is an autosomal recessive disorder caused by mutation in TBCE gene, that encodes one of the chaperone proteins necessary formation of α–β-tubulin heterodimers (Parvari et al. 2002). Affected patients can present susceptibility to recurrent bacterial infections, especially pneumococcal infections, sepsis, or relevant skeletal involvement (Hershkovitz et al. 2004). Immunological alterations found in patients with HRD are impairment of chemotactic migration and phagocytosis of PMN (Hershkovitz et al. 2007).

It is noteworthy that infections, in case of glandular involvement, can also cause PTH alterations. In this sense the link between infections and PTH dysfunction is a two-way path. In particular, it has been described that infection by M. tuberculosis can present as hyperparathyroidism (Mayo-Yanez et al. 2020; Kar et al. 2001; Singh et al. 2016). In a study describing 102 autopsy cases of patients affected by HIV, parathyroid hyperplasia was the most common histological alteration, with an higher frequency than controls (Cherqaoui et al. 2014). Beyond these histological examinations, another clinical study has demonstrated that HIV-affected patients showed lower levels of PTH than controls (Hellman et al. 1994), and both basal and EDTA stimulated PTH secretion is altered in HIV infected patients (Jaeger et al. 1994). On the contrary, parathyroid infection can also lead to glandular hypofunction. Interestingly, new onset hypoparathyroidism has been described also in a patient infected by Sars-Cov2 (Elkattawy et al. 2020).

8 Conclusions and Future Directions

Parathyroid disorders are characterized by alterations of calcium metabolism and non-physiological levels of PTH. Patients with hyper- and hypoparathyroidism are exposed to a higher risk of infectious diseases, increasing morbidity, and mortality. Throughout the last decades, many attempts in unveiling the pathophysiology of PTH and calcium metabolism disorders have been made, with conflicting results, and data from randomized trials are lacking. From our review emerged that hyperparathyroidism is associated to imbalances both in innate and in acquired immune response.

However, published studies have many limitations: most are on patients affected by hyperparathyroidism secondary to CKD, in which factors other than PTH can affect immune response, while other are *in vitro* studies or on animal models, and most of them were conducted with very heterogenous methods, compromising the reliability of results.

Therefore, trials on primary hyperparathyroidism and on the difference between patients who undergo surgical intervention (which is able to normalize PTH) compared to patients who receive medical therapy (which is able usually only to normalize calcium levels without normalizing PTH) might provide further explanation for the underlying mechanisms and long-term prognosis. Moreover, studies on normocalcemic hyperparathyroidism could highlight the potential detrimental role of high levels of PTH, independently from hypercalcemia.

In hypoparathyroidism, we have strong epidemiological data that show an increased risk of infections in hypoparathyroidism. mechanisms underling this condition are understudied, but a recent trial showed significant alterations in monocytes and $CD4^+$ lymphocytes. As in hyperparathyroidism, though, available studies are not powered enough to distinguish between the effects of PTH and those of concomitant biochemical alterations. Trials comparing conventional replacement therapy (calcium and calcitriol) and replacement therapy with PTH (PTH 1-84 or PTH 1-34) should therefore also include immunological evaluations in order to analyse the potential benefits of PTH replacement on infectious diseases and immune alterations. Finally, the evidence acquired so far is strong enough to suggest a major role for PTH in immune homeostasis and to prompt further translational studies.

In the era of precision medicine and tailored therapeutic approaches, the role of immune system as a biomarker of health and disease is increasing. Exploring the role of PTH on immune cells will help understanding the observed alterations in parathyroid disorders, evaluate the role of PTH replacement in preserving immune function in hypoparathyroidism and ultimately improve the quality of life and long-term longevity of patients with parathyroid disorders.

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In Vitro Antimicrobial Susceptibility Testing of Biofilm-Growing Bacteria: Current and Emerging Methods

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Abstract

The antibiotic susceptibility of bacterial pathogens is typically determined based on planktonic cells, as recommended by several international guidelines. However, most of chronic infections – such as those established in wounds, cystic fibrosis lung, and onto indwelling devices – are associated to the formation of biofilms, communities of clustered bacteria attached onto a surface, abiotic or biotic, and embedded in an extracellular matrix produced by the bacteria and complexed with molecules from the host. Sessile microorganisms show significantly increased tolerance/resistance to antibiotics compared with planktonic counterparts. Consequently, antibiotic concentrations used in standard antimicrobial susceptibility tests, although effective against planktonic bacteria *in vitro*, are not predictive of the concentrations required to eradicate biofilm-related infections, thus leading to treatment failure, chronicization and removal of material in patients with indwelling medical devices.

Meeting the need for the *in vitro* evaluation of biofilm susceptibility to antibiotics, here we reviewed several methods proposed in literature highlighting their advantages and limitations to guide scientists towards an appropriate choice.

Keywords

Antibiotic resistance · Antibiotic therapy · Biofilm-related infections · Susceptibility tests · Treatment failure

Abbreviations

CLSI	Clinical and Laboratory Standards Institute
EUCAST	European Committee on Antimicrobial Susceptibility Testing
CF	Cystic fibrosis
AST	Antibiotic susceptibility testing
MTP	Microtiter plate
CBD	Calgary biofilm device
OD_{650}	Optical density at 650 nm
ASTM	American Society for Testing and Materials International
BRT	BioFilm® ring test
DFBR	Drip flow biofilm reactor

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MBIC	Minimum Biofilm Inhibitory Concentration
BPC	Biofilm-Prevention Concentration
BBC	Biofilm Bactericidal Concentration
MBEC	Minimum Biofilm Eradication Concentration
MIC	Minimum Inhibitory Concentration
PYO	Pyocyanin
IMC	Isothermal microcalorimetry
CVC	Central venous catheter

1 Background

The susceptibility of a bacterial pathogen to antibiotics is typically determined based on planktonic cells, as recommended by CLSI and EUCAST international guidelines. Nevertheless, successful treatment of chronic infections, such as a pulmonary infection in cystic fibrosis (CF) patients and those related to indwelling devices, usually requires the eradication of the pathogen growing in a biofilm (Hauser et al. 2011; Tande and Patel 2014).

Current definitions have described biofilms as a functional consortium of microorganisms attached to each other and onto biotic or abiotic surfaces, embedded in an extracellular matrix produced by the bacteria and complexed with other components derived from the host.

The treatment of biofilm-related infections is difficult, as sessile microorganisms are inherently tolerant/resistant to antibiotics compared with their planktonic counterparts (Stewart and William Costerton 2001; Caraher et al. 2007; Molina-Manso et al. 2013a, b; Otter et al. 2015; Luo et al. 2020). Because of this, the antibiotic concentrations used in standardized antibiotic susceptibility testing (AST), although effective against planktonic bacteria *in vitro*, are not predictive of those required to eradicate biofilms at the site of infection. This leads to multiple rounds of antibiotics, treatment failure, chronicization of infection and the removal of materials in patients with indwelling medical devices (Widmer et al. 1990; Smith et al. 2003; Hola et al. 2004; Kathju et al. 2014;). To make the picture even more complex, antibiotics at sub-inhibitory concentrations can also stimulate biofilm formation, further confusing the issue of appropriate treatment (Rachid et al. 2000; Wu et al. 2014).

There is, therefore, an urgent need for dedicated laboratory technologies to accurately assess, during diagnostic testing, the susceptibility of biofilms to antimicrobial agents. In this report, several models proposed over the last decade for the *in vitro* evaluation of the antimicrobial activity against biofilms are reviewed. Furthermore, the parameters that should be considered in the development of experimental protocols for the study of the efficacy of antibacterial agents against pathogenic bacteria in biofilms are also discussed.

2 Laboratory Models for *In Vitro* Biofilm Antibiotic Susceptibility Testing

The constant increase in the number of laboratory methods recently proposed for assessing the susceptibility of biofilms to antibiotics clearly indicates the demand for techniques alternative to the classic antibiotic susceptibility tests.

The biofilm-based growth models can be classified as closed (batch culture) or open (continuous culture) systems, based on nutrient delivery. The selection of the optimal model depends on the clinical setting there is need to mimic, considering the fact that this might require combining different approaches. The main features, along with the relative advantages and limitations, of each model proposed over the last decade for the *in vitro* evaluation of biofilm susceptibility to antibiotics are described below and summarised in Table 1.

Table 1 Main features of *in vitro* models recently proposed in the literature for assessing the *in vitro* susceptibility of biofilm to antibiotics

	Closed			Open			
Features	Microtiter plate	Calgary biofilm device	BioFilm® ring test	Flow cell	Microfluidic systems	CDC biofilm reactor	Drip flow biofilm reactor
High throughput testing	Yes	Yes	Yes	Not	Yes	Not	Not
Cost	+	++	++	+++	++++	++++	+++
Dedicated instruments	Not	Not	Yes	Yes	Yes	Yes	Yes
Exhaustion of nutrients	Yes	Yes	Yes	Not	Not	Not	Not
Single use	Yes	Yes	Yes	Not	Not	Not	Not
Reproducibility	+	++	++	++	++	++	+
Sensitivity	+	+	+++	++	++	++	++
Time to results	+	+	++	+	+	+	+
Aggregation	Possible	Not	Not	Not	Not	Not	Not
Endpoint measurement	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Microscopic observation	Yes	Yes	Not	Yes	Yes	Yes	Yes
Biofilm viability assessment	+	++	+	+	++	++	++
Combinatorial approach[a]	+++	+++	Not	+	+	+	+
Contamination	++	++	Not	+	++	+	+
Standardized	Yes	Yes	Not	Not	Not	Yes	Yes
Published biofilm-based AST studies	++++	+++	+	+++	+	++	+

[a]Combinatorial approach: challenge plate configurations can be set up to perform checkerboard assays to identify antimicrobial antagonism or synergy (Harrison et al. 2008), and to perform multiple combination susceptibility testing (Slinger et al. 2006)

2.1 Closed ("Batch Culture" Based) Models

In these models, the microorganisms with relatively fast growth rate are incubated in a closed vessel with a single batch of medium warranting a limited amount of nutrients (McBain 2009; Azeredo et al. 2017). The ease of use, reproducibility and applicability in high-throughput analysis make these models easily implemented in a microbiology laboratory's routine and useful for high-throughput analysis.

2.1.1 Microtiter Plate Method (MTP)

In this assay, bacteria are incubated in the "U"-bottom wells of a polystyrene 96-well microtiter plate containing sterile growth medium (Stepanović et al. 2007; Azeredo et al. 2017). Following incubation, planktonic bacteria are rinsed away, and the remaining adherent bacteria (biofilms) are quantified.

Biofilm measurement is generally performed by measuring the optical density after staining biofilm with crystal violet (Stepanović et al. 2007; Azeredo et al. 2017). Although rapid, easy to perform and reproducible, this technique is not informative for biofilm viability, but rather for biofilm biomass only. Indeed, the cationic dye stains the negatively charged biofilm constituents such as cells, in a non-specific way, regardless of their viability and extracellular matrix. The residual viability of a biofilm after exposure to an

antimicrobial agent can be accurately measured using a viable cell count assay, after detaching biofilm by scraping or sonication. Other less laborious but also less reliable approaches use "metabolic" stains directly on biofilm or the cells collected after its disruption: blue phenoxazin dye resazurin (Wannigama et al. 2020), tetrazolium salts (Sabaeifard et al. 2014), Alamar blue (Kim et al. 2010a, b), and fluorescent chromophores (e.g., Syto-9, propidium iodide) (Müsken et al. 2010).

MTP method was also used to combine the bacterial viability staining with automated confocal laser scanning microscopy, thus allowing easy qualitative and quantitative evaluation of biofilms after exposure to antibiotics (Müsken et al. 2010).

User-friendly, rapid and cost-saving (using small volumes of antimicrobials and minor media consumption) MTP is one of the most widely used models for testing biofilm susceptibility to both antibiotics and disinfectants. It allows to evaluate the efficacy of various concentrations of test compounds in preventing or eradicating biofilms (Pitts et al. 2003; Takahashi et al. 2007; Božić et al. 2018). Moreover, the possibility of testing multiple replicates with a low operating volume makes MTP commonly used for screening large drug libraries (Van den Driessche et al. 2017; Gilbert-Girard et al. 2020). However, a major drawback of this method is the nutrient depletion during the incubation period, with a significant impact on the biofilm susceptibility towards the antibiotics (Manner et al. 2017). Furthermore, portions of the biofilm biomass may stem from cells sedimented to the bottom of the wells, rather than being the result of a biofilm-forming process.

2.1.2 Calgary Biofilm Device (CBD)

In this method, the biofilm can form – under dynamic (rocking at 20 Hz) or static conditions – onto the surface of polystyrene pegs present on the lower surface of a coverlid that fit into the wells of the microtiter plate containing the growth medium and bacteria (MBEC™ Assay procedural manual, version 2.1.; Ceri et al. 1999). The peg lids are then rinsed and placed onto flat-bottomed microtiter plates, where they are exposed (18-20 h, 37 °C) to different antibiotic concentrations (MBEC™ Assay procedural manual, version 2.1.; Ceri et al. 1999). The peg lids are rinsed and placed into an antibiotic-free medium in a flat-bottomed microtiter plate where the biofilm is recovered after detaching it by light centrifugation/sonication (MBEC™ Assay procedural manual, version 2.1.; Ceri et al. 1999).

The biofilm viability residual after antibiotic exposure can be evaluated by (MBEC™ Assay procedural manual, version 2.1.; Ceri et al. 1999, Harrison et al. 2010): i) a visual check of wells for turbidity; ii) measuring optical density at 650 nm (OD_{650}) before and after 6 h-incubation at 37 °C considering a mean OD_{650} difference of $\geq$0.05 as adequate biofilm growth for the positive control wells; or iii) viable cell count.

CBD has been employed to perform AST of biofilm formed by enterococci (Sandoe et al. 2006), *P. aeruginosa* from CF patients (Hill et al. 2005; Høiby et al. 2019), *S. aureus* causing prosthetic infections (Molina-Manso et al. 2013a, b; Revest et al. 2016), *Burkholderia pseudomallei* (Anutrakunchai et al. 2015), and to compare efficacies of multiple antibiotic combinations against *P. aeruginosa* biofilm (Moskowitz et al. 2004; Tre-Hardy et al. 2008; Díez-Aguilar et al. 2017). In addition, CBD has been the first approved ASTM (American Society for Testing and Materials International) standardized biofilm disinfectant efficacy test method (Parker et al. 2014).

2.1.3 BioFilm® Ring Test (BRT)

Its functioning principle is based on the potential immobilization of magnetic microbeads by bacteria forming a biofilm in the well bottom of a modified 96-well polystyrene microplate (Chavant et al. 2007). Biofilm-associated adherence is determined when beads remain scattered after the application of a magnetic field; on the contrary, in the presence of planktonic cells the beads are immobilized in the centre of the well bottom (Liesse Iyamba et al. 2011; Puig et al. 2014).

BRT has been used to assess the ability to form biofilm by non-typeable *H. influenzae* strains

(chronic obstructive pulmonary disease, otitis media, pneumoniae), *S. aureus* and *S. epidermidis* (acute and chronic osteomyelitis, infectious arthritis), and *P. aeruginosa* CF strains (Valour et al. 2013; Valour et al. 2015; Olivares et al. 2016). An alternative protocol of the BRT, the clinical Biofilm Ring Test, can provide an accurate and rapid measurement of biofilm formation for the most common pathogenic bacteria seen in clinical practice (Di Domenico et al. 2016).

A recent, not yet standardized, extension of BRT is Antibiofilmogram®, which was tested for susceptibility profile testing of bone and joint infection-related *S. aureus* and *P. aeruginosa* CF biofilms, and could be of great interest after surgical operations on contaminated prostheses and after bacteremia to prevent the colonization of the device (Tasse et al. 2016).

The primary advantage of this methodology is the possibility to get results within a pair of hours; however, it allows for the evaluation of the adhesion, the initial step of biofilm, and requires a dedicated scanning plate reader.

2.2 Open ("Continuous Culture" Based) Models

In these models, microorganisms grow at a controlled rate and the nutrients are provided via continuous media flow (McBain 2009; Azeredo et al. 2017). The possibility of replicating *in vivo* conditions through the control of growth parameters and dynamics such as nutrient delivery, flow, and temperature, makes open models useful for the in-depth study of biofilm formation. However, they are hard to implement in a classic diagnostic workflow.

2.2.1 Flow Cell-Based Models

Biofilm formation is allowed in a capillary, onto coupon or glass slide. They enable a non-destructive, real-time, microscopic observation of the antibiotic effect against biofilm (Heydorn et al. 2000; Klausen et al. 2003; Pawley 2006; Haagensen et al. 2007). Biofilm viability can be assessed by fluorescence (e.g., Live/Dead viability kit tags live bacteria with green/yellow fluorescent proteins, whereas dead ones with propidium iodide). In addition, structural parameters such as biomass, average and maximum thickness and roughness coefficient can be measured by a dedicated software (e.g., COMSTAT) to assess the time course and spatial activity of antibiotics (Heydorn et al. 2000). Viable cells and antibiotic-resistant mutants can also be determined by cell viable count after detaching biofilms by washing the channels with glass beads in NaCl (Macià et al. 2011).

Completely autoclavable and therefore re-useable, flow cell-based models are particularly indicated to evaluate new approaches to control biofilm-associated wound infections. Indeed, "Gram-negative shift" – a well-known *in vivo* phenomenon in this kind of infections – occurs only under flow conditions (Alves et al. 2018).

It has been used for AST of biofilm formed by methicillin-resistant *S. aureus* (Smith et al. 2013), *P. aeruginosa* (Mettrick et al. 2020), and *P. gingivalis* (Asahi et al. 2012) as well to test the efficacy and the use of biocides to eliminate pathogens in the health care environment (El-Azizi et al. 2016). A flow-cell apparatus irrigated with an artificial CF sputum medium has been proposed for the mathematical modelling of *P. aeruginosa* biofilm treatment in CF lung (Miller et al. 2014).

2.2.2 Microfluidic Systems

The smaller volumes, inherent in microfluidic devices, along with the ability to produce multiple concentration gradients provided a faster alternative to current AST.

Quantification of viability after exposure to antibiotics is performed by measurement of fluorescence (using GFP-tagged bacteria or Live/Dead staining) (Kim et al. 2010a, b, 2012a). While these models are robust and promising, they require expensive optical equipment and genetically modified bacteria or selective labels (Richter et al. 2007; Holman et al. 2009; Yawata et al. 2010).

Microfluidics-based devices, including the relatively recent BioFlux™ device, are fully integrated platforms consisting of modified 96-well plates

with laminar flow chambers, a shear-flow control system, an imaging system, and advanced software for data collection and analysis; used to test the activity of antibiotics, alone and in combination, against biofilm formed by *P. aeruginosa, Staphylococcus pseudintermedius,* and *E. coli* (Webster et al. 2015; Kim et al. 2012b; Díez-Aguilar et al. 2017).

2.2.3 CDC Biofilm Reactor

Biofilms develop on coupons suspended from the lid and immersed in a growth medium. Antimicrobial agents can be added to the bulk fluid phase, simultaneously exposing all coupons. Sampling is achieved by removing the coupon holder at desired times, then coupons are sonicated, and finally vigorously vortexed to dislodge and disperse the cells from the biofilm. Biofilm measurement can be performed by viable cell plate counting or CLSM staining (Fjeld and Schuller 2013; Kim et al. 2008).

CDC biofilm reactor is a standardized device, particularly indicated for modelling prosthesis-related (allowing for high flexibility in choosing material) (Hall Snyder et al. 2014), and oral (operating under higher shear stress) (Siala et al. 2018) biofilm AST.

This model has been used to assess the efficacy of both antibiotic and antimicrobial dressings against mono- and polymicrobial *S. aureus*/*P. aeruginosa* biofilms (Miller et al. 2020; Suleman et al. 2020), as well as for assessing antibiotic susceptibility of biofilm by vancomycin-resistant *Enterococcus faecium* (Jahanbakhsh et al. 2020).

In addition, the CDC reactor has recently been used to assess the pharmacokinetics/pharmacodynamics of beta-lactams in continuous infusion for biofilm infections by *P. aeruginosa* (Gomez-Junyent et al. 2020).

2.2.4 Drip Flow Biofilm Reactor (DFBR)

It consists of several, separate, parallel channels, each one equipped with an individual lid to keep the aseptic conditions during the sampling process (Manner et al. 2017). The medium enters in each chamber, containing a coupon that may be made of a variety of materials, through a 21-gauge needle inserted in the lid septum. During operation, the reactor is maintained under low shear conditions, namely at an angle of 10° with the medium running down the length of the coupons (Manner et al. 2017).

DFBR is designed to assess the efficacy of disinfection strategies for biofilm control and removal (Curtin and Donlan 2006; Ledder et al. 2010; Ammons et al. 2011). Standardized, this model is suitable to mimic the biofilm growth occurring at the air/liquid interface under low shear stress situations, as is the case of CF lungs, teeth biofilms and wounds (Bird et al. 2002; Carlson et al. 2008; Brambilla et al. 2014). Furthermore, it allows for a generalized conceptual model of biofilm antimicrobial tolerance, with the establishment of concentration gradients in metabolic substrates and products, and biofilm cell dormancy (Stewart et al. 2019).

However, the main drawbacks of DFBR are laboriousness, the need for large volumes of media and test samples, and the limited number of antimicrobials (usually 4–6) that can be tested in parallel.

2.2.5 Robbins Device

This device consists of a pipe with several threaded holes where coupons are mounted on the end of screws placed into the liquid stream. The coupons are aligned parallel to the fluid flow and can be removed independently.

The main advantage is the possibility to sustain continued biofilm growth, for several weeks or more, without interruption (Teodosio et al. 2011; Teodosio et al. 2012). However, it relies on unidirectional shear, and requires intermittent sterilization, which limits throughput and risks contamination; in addition, artefacts due to the handling of the samples, and entry effects are common, thus requiring a stabilization length to allow for direct comparison of the biofilm formed onto different coupons.

The Robbins device was mainly used for testing the susceptibility to antibiotics of biofilms formed by several bacterial species in the oral cavity (Larsen and Fiehn 1995; Honraet and Nelis 2006; Blanc et al. 2014; Lasserre et al. 2018), on central venous catheters (Mekni et al. 2015), and voice prostheses (Free et al. 2003).

3 Quantifying the Antibiotic Activity Against Biofilms

3.1 Pharmacodynamic Parameters

Using both open and closed models, several pharmacodynamic endpoints can be obtained to finally evaluate the efficacy of an antibiotic against biofilm-growing bacteria (Macià et al. 2014). The ability of an antibiotic to affect biofilm formation is commonly evaluated by calculating either the Minimum Biofilm Inhibitory Concentration (MBIC) or the Biofilm-Prevention Concentration (BPC). MBIC can be measured both by spectrophotometry and cell viable counts. In the first case, MBIC is defined as the lowest concentration of an antibiotic causing an OD_{650} reduction of at least 10% compared to the unexposed control well readings (Moskowitz et al. 2004). Regarding cell counts, the MBIC is the lowest concentration of an antimicrobial at which there is no time-dependent increase in the mean number of biofilm viable cells when an early exposure time is compared with a later exposure time. BPC is defined as the lowest concentration that prevents biofilm formation by reducing the cell density, and its measurement requires that biofilm be exposed to an antibiotic and tested at different concentrations, during its formation (Kolpen et al. 2010).

Both MBIC and BPC endpoints are particularly relevant in the case of CF patients where the early stage of colonization by *P. aeruginosa* can be effectively eradicated by adopting an appropriate antibiotic therapy (Cantón et al. 2005).

The effect of an antibiotic against preformed (mature) biofilms can be measured by calculating either the Biofilm Bactericidal Concentration (BBC) or the Minimum Biofilm Eradication Concentration (MBEC) (Girard et al. 2010; Macià et al. 2014; Brady et al. 2017). BBC is defined as the lowest concentration of an antibiotic that is able to cause a 99.9% reduction in the cell viable count of a biofilm culture as compared to the unexposed growth control. MBEC is defined as the lowest concentration of an antibiotic that is required to eradicate the biofilm, namely the lowest antibiotic concentration preventing visible growth in the recovery medium used to collect the biofilm sample.

3.2 What Does It Mean a "Significant Reduction" for Medically Relevant Biofilms?

There is need to define a "target" reference value to accurately ascertain the effectiveness of an anti-biofilm treatment. This would provide a useful guide to clinicians that generally manage infections choosing the most relevant and effective agent based on planktonic paradigms. Deciphering what may be a "target reference", there are two points of view to consider when posing questions around the performance standards of an agent that cites claims on "efficacy" or "effectiveness". First, there is a regulatory perspective that looks to determine a "target reference" based on standardized approaches using statistical attributes. Secondly, it is needed to understand how well results from *in vitro* studies could translate to clinical efficacy and if the adoption of the selected target references might lead to improvements in clinical symptoms until the resolution of chronic infections.

Despite the fact that the studies that compared clinical isolates using biofilm AST were focused on a small number of species (mainly *S. aureus, S. epidermidis, P. aeruginosa*, and *E. coli*), it has been suggested that treatment decisions should be based on MBIC or MBEC values (Brady et al. 2017).

Unfortunately, to date, there is no consensus on what a potential target value could be due to contrasting or insufficient scientific evidence. Indeed, two randomized controlled clinical trials that addressed the treatment of *P. aeruginosa* infections in CF patients have reported that MBEC value does not demonstrate the superiority of treatment based on biofilm AST over the conventional AST (Waters and Ratjen 2017). The authors suggested that biofilm-based AST may be more appropriate to define alternative, more

effective, formulations of drugs that can be tested in clinical trials.

In addition, MBIC value does not predict a successful clinical outcome for the treatment of catheter-related bloodstream enterococcal infections (Sandoe et al. 2006).

The absence of a "target" reference value required to ascertain the efficacy of an antibiofilm therapy suggests that a complete eradication is required. This conservative approach might over-simplify the real situation, since a target reference value may vary depending on the infecting strain, the type of infection, and the immune status of the patient.

4 When Is Biofilm AST «Justified»?

Alternatively, biofilm-based susceptibility testing should be justified only if the results cannot be predicted based on current microbiological characterization, and if their interpretation will provide clinical benefit.

The use of biofilm AST revealed that antimicrobial susceptibilities based on biofilm growth differ significantly from those based on planktonic growth. Several studies have reported BIC and MBEC values that were significantly higher (100 to 1000 times) than their corresponding Minimum Inhibitory Concentration (MIC) values (Smith 2005; Høiby et al. 2010; Kostakioti et al. 2013). Furthermore, Moskowitz et al. (2005) observed that the adoption of biofilm AST led to substantially different simulated regimens for *P. aeruginosa* CF airway infection when compared with conventional testing, with only 40 and 20% of chronic and acute regimens, respectively, consisting of drugs in the same two mechanistic classes by both methods.

Unfortunately, the current scientific evidence is insufficient to recommend the choosing of antibiotics based on biofilm AST rather than conventional AST. In this frame, a recent systematic review compared biofilm AST-driven therapy to conventional AST-driven therapy in the treatment of *P. aeruginosa* infection in CF patients (Waters and Ratjen 2017). The searches identified two multicenter, randomized, double-blind controlled clinical trials where BIC values were assessed by CBD. The authors found no difference in any of the selected outcomes (i.e., forced expiratory volume at one second, time to next exacerbation, adverse events, sputum density, quality of life) between the two groups (biofilm-based versus conventional AST) in either trial (Waters and Ratjen 2017).

5 Why Is *In Vitro* Biofilm AST Not Predictive of the *In Vivo* Situation?

Most of our knowledge regarding biofilm susceptibility to antibiotics are derived from *in vitro* assays, although they are often poor representatives of the "environment" observed at the infection site (Bjarnsholt et al. 2013). The lack in the clinical predictive value of biofilm AST might, therefore, be the consequence of an oversimplification of the bacterial growth conditions at the infection site.

Biological features should be considered in *in vitro* testing to mimicry the physicochemical conditions faced *in vivo* by an antibiotic are summarized in Table 2.

The antibiotic susceptibility of biofilms is typically performed under favourable conditions, namely in nutrient-rich media and without any apparent stressors such as adverse pH, O_2 tension, osmolarity or nutrient availability. Additionally, host defense mechanisms (cellular and antibody) are lacking in all proposed models since they are difficult to reproduce. The environments in which biofilms develop *in vivo* can be vastly different: several unfavourable conditions trigger adaptive mechanisms with consequent modification of both the local environment and the microorganism phenotype (metabolic rate, protein production, cellular replication, and expression of surface proteins) so that local conditions in the biofilm allow for microorganism survival (Koch and Hoiby 1993; Gibson et al. 2003; Jesaitis et al. 2003; Bjarnsholt et al. 2013; Campbell et al. 2014; McLaren and Shirtliff 2015).

Furthermore, biofilm structures observed *in vivo* often differ from those obtained in

Table 2 Biological and technical features responsible for the poor predictivity of biofilm AST

Biofilm features	***In vivo***	***In vitro***
Test medium	Sputum, saliva, urine, blood	Commonly used, rich, culture media
Flow type	Mostly dynamic	Mostly static
Exposure to nutrients	Not always exposed to a continuous flow of fresh media or suspended in static liquid	Continuous exposure
Exposure to antibiotics	Indirectly reached by antibiotics	Direct exposure
Adhesion to a surface	Not always; biofilm can be embedded in tissue or sited between implant and tissue	Mostly attached to a surface
Organization	Can be non-attached relatively small aggregates	"Mushroom-like" structure
Atmosphere	Mostly hypoxic or anoxic	Mostly aerobic
Etiology	Often polymicrobial	Mostly monomicrobial
Host response	Inflammatory response elicited	Not considered
Microbial diversity	Can be high (polymicrobial)	Mostly monomicrobial; underestimated (isolates selected due to prevalence or specific monotypes)
Technical features	***In vivo***	***In vitro***
Antibiotic neutralization		Mostly not performed
Cell recovery by viable count		Incomplete or underestimated (sonication; antibiotic carry-over)
Endpoint for treatment effectiveness	Possible time-dependent effect	Mostly 24 h
Adequate clinical outcome	Antibiotics might cause planktonically shed bacteria from biofilm, therefore not lead to a decreased bacterial density	Decrease in bacterial sputum is the most common outcome chosen to assess CF therapy effectiveness

in vitro studies, in terms of physical dimensions and microenvironments (Bjarnsholt et al. 2013; Roberts et al. 2015). Lastly, biofilm-based chronic infections have often polymicrobial aetiology and are characterized by high microbial diversity, whereas *in vitro* AST assays are commonly performed using monomicrobial biofilm formed by isolates selected due to the prevalence of specific monotypes (Wolcott et al. 2013).

On the other hand, technical variables could also account for the poor predictivity of biofilm AST (Melchior et al. 2007; VanDevanter et al. 2011) (Table 2).

An overestimation of the anti-biofilm activity of an antibiotic might be due to a "carry-over" effect, that is a continued antibiotic activity against biofilm during incubation and microbial recovery. This can be avoided by using a neutralizing agent or diluting the antibiotic to a sub-inhibitory level (e.g., by rinsing the biofilm prior to recovery) (Russel et al. 1979).

Sub-optimal biofilm cells recovery, during and/or after their detachment, could underestimate the efficacy of an antibiotic treatment. A highly conservative collection of adhered biofilm biomass without interfering with adhered cells viability can be achieved by using an ultrasonic cleaner, after standardization of the sonication time and power followed by a collection of microorganisms on the device by sampling the media after sonication, and finally by serial dilution and spread or spot plating to count the individual colonies plated (Harrison et al. 2010; ASTM International Standard 2012; Incani et al. 2015).

MBEC is typically determined *in vitro* by exposing biofilm to antibiotics for 24 h or less. However, biofilm-based infections are difficult to treat, especially because persister cells, tolerant to systemic levels of antimicrobials, might repopulate the biofilm when antibiotic therapy is discontinued (Lewis 2007). Confirming this,

several clinical trials have demonstrated that the chance of a positive therapy outcome increases with a longer duration of the therapy (Castaneda et al. 2016). Therefore, one-day assays for MBEC may overestimate the local antimicrobial levels needed to kill organisms in a biofilm if local levels are sustained for longer than 24 h, thus requiring the development of the extended MBEC assay.

Finally, both adequate clinical (VanDevanter et al. 2011) and technical (Melchior et al. 2007) endpoints should be also considered for a reliable assessment of the treatment efficacy.

6 Bridging the Gap Between *In Vitro* and *In Vivo* Biofilms: Beyond Commonplace AST Platforms

In trying to close the gap between *in vitro* and *in vivo* biofilm modelling, other more complex laboratory models have been recently proposed in the literature for studying biofilm formation and physiology. These models could represent new platforms for a reliable assessment of biofilm AST.

6.1 Poloxamer Thermo-Reversible Matrix

Poloxamers are nonionic triblock copolymers composed of a central hydrophobic chain of polyoxypropylene (poly(propylene oxide)) flanked by two hydrophilic chains of polyoxyethylene (poly(ethylene oxide)).

Microorganisms cultured in a semi-solid poloxamer matrix – such as the nontoxic and inert Poloxamer 407 – form microcolonies that exhibit a biofilm phenotype with increased tolerance to disinfectants, antimicrobials, and silver-containing wound dressings (Gilbert et al. 1998; Clutterbuck et al. 2007; Percival et al. 2007; Yamada et al. 2011; Taylor et al. 2016). Antimicrobial efficacy was measured by fluorescence (e.g., Syto-9 Live/Dead stain) or viable cell count.

The main advantages consist of an easy and improved biofilm recovery after poloxamer liquefaction by "flash cooling" (liquid at <15 °C, poloxamers become a semisolid gel at higher temperatures); in addition, the biofilm is not attached to a surface, as observed in CF lung infections, and chronic wounds (Bird et al. 2002; Carlson et al. 2008; Bjarnsholt et al. 2013; Brambilla et al. 2014).

6.2 Chip Calorimetry

This method enables the detection of microorganisms via their metabolic heat and can be applied for the real-time monitoring of biofilm activity. The main component is a silicon chip, equipped with a thermo-sensitive membrane containing 118 BiSb/Sb thermocouples to convert the heat generated by the bacteria into a voltage signal. A flow-through system is required to avoid bias due to the planktonic cells.

A chip-calorimeter has been validated for *Pseudomonas putida* biofilm AST, comparatively to ATP content measurement and cell viable count (Buchholz et al. 2010; Mariana et al. 2013).

This method has the potential for multichannel chip-calorimetry (the measurement of separated samples with one calorimeter) or calorimetric reading of microtiter plates. Furthermore, it does not require biofilm disruption or recultivation (long-term, real-time monitoring), has a small size (high flexibility, low medium consumption), and can be informative about the antibiotic mechanism of action.

6.3 Microfluidic-Electrochemical Coupled System

A cheaper and easier method of determining the relative number of live cells in a biofilm under exposure to antibiotics can be achieved by monitoring the electrochemical response of the system.

P. aeruginosa produces the blue electro-active molecule pyocyanin (PYO), a potential marker of cell viability and virulence (Usher et al. 2002; Allen et al. 2005). PYO undergoes reversible

redox (exchange of electrons) reactions, and its presence can be therefore measured with standard electrochemical techniques (Sismaet et al. 2014).

In this way, the decreased current response is directly related to a decrease in the measured PYO, indicating a correlation between the antibiotic concentration and PYO production.

The main advantage is that an electrochemical sensor might be used for monitoring the status of infections *in vivo* while carrying out antibiotic treatment.

6.4 Alginate Bead System

In this method, the biofilm can grow into alginate beads to obtain spatially structured aggregates like those seen in CF lungs and chronic wounds (Bjarnsholt et al. 2013; Kragh et al. 2014). Alginate-encapsulated bacteria are indeed metabolically less active – and, therefore, more tolerant to antibiotics – separated by a secondary matrix, and not attached to a surface. Moreover, a steep O_2 concentration gradient is generated within the alginate beads, with the possibility to incorporate the alternative electron acceptor NO_3- into the beads, to mimic the *P. aeruginosa* anaerobic growth.

The alginate bead model was mainly used to test *P. aeruginosa* biofilm resistance to disinfectants, such as hydrogen peroxide and monochloramine (Cochran et al. 2000), and antibiotics (Cao et al. 2015; Cao et al. 2016).

Recently, Dall et al. (2017) presented a dissolving alginate bead model utilizing a sodium alginate substratum for surface biofilm colony formation, which can be readily dissolved for an accurate evaluation of viable organisms after an antibiotic challenge.

6.5 Duckworth Biofilm Device

This device is a single part instrument, consisting of individual channels (Duckworth et al. 2018). Biofilms can be cultured on cellulose acetate/cellulose nitrate disks for recovery and enumeration, or on glass coverslips for microscopic analysis.

The main advantages are as follows: re-usable (sterilizable), technical expertise not required, no cross contamination, can be 3D-printed in a variety of materials, throughput, multi-sample analysis.

Particularly indicated for chronic wound biofilms, which are typically not submerged but grow at the air-liquid interface of the wound bed, being "fed" from beneath by wound exudate (Duckworth et al. 2018). This approach also allows for the application of wound dressings (Duckworth et al. 2018).

6.6 3D Collagen-Containing Matrix Wound Model

In this model, the biofilm grows as cell aggregates into a collagen gel matrix with serum protein mimicking the wound bed of chronic wounds (Werthén et al. 2010; Pompilio et al. 2017). The model comprised important hallmarks of biofilms including microcolonies embedded in a self-produced, extracellular polymeric matrix, increased antibiotic tolerance, and the host defence (Werthén et al. 2010). Furthermore, the developed bacterial aggregates in the collagen matrix resembled those observed in real chronic wounds thus indicating the relevance of the model (Werthén et al. 2010).

This model has been used to test the susceptibility to antibiotics of biofilm by common (*S. aureus* and *P. aeruginosa*) (Werthén et al. 2010), as well as infrequent (*Myroides odoratimimus*) (Pompilio et al. 2017) colonizers of chronic wounds.

6.7 Isothermal Microcalorimetry (IMC)

IMC allows for real-time monitoring of bacterial viability based on the metabolism-related heat production (Braissant et al. 2010). Biofilms are formed onto a substrate in a sealed glass ampoule placed in a microcalorimeter for real-time measurement of heat flow and heat.

IMC has been mainly used for testing the *in vitro* and *in vivo* activities of different antibiotic formulations on biofilms formed by Gram-positive (Gonzalez Moreno et al. 2017; Butini et al. 2019a; Butini et al. 2019b), Gram-negative (Wang et al. 2019) and mycobacteria (Solokhina et al. 2018). It also finds application for quantifying the antimicrobial efficacy of implant coatings and the study of the antibiotic eluting kinetics from different biomaterials (Butini et al. 2018; Santos Ferreira et al. 2018).

Although IMC does not allow for direct quantification of the non-replicating cells in the biofilm or the total biomass, the use of this technique shows several advantages. Samples are undisturbed and then can be reused for further analyses. Furthermore, it allows for a high-sensitive (detection limit 10^4–10^5 CFU/ml), fast (results are available within hours) and reliable investigation of biofilms. Indeed, although not yet standardized, IMC has been demonstrated to generate data in agreement with those obtained after performing standard conventional tests (Mihailescu et al. 2014; Gonzalez Moreno et al. 2017; Butini et al. 2018; Di Luca et al. 2018).

7 Choosing a Reliable *In Vitro* Model for Biofilm AST

Many laboratory biofilm models are available to assess the AST of biofilms, from "batch culture-based" models to "continuous culture-based" models. Their complexity varies considerably – increasing from "batch" to "continuous" models – making the choice very difficult.

Biofilm antibiotic efficacy studies should be assessed using one or more (combinatorial approach) models mimicking the conditions of its clinical application.

Indeed, the selection of the model system can have a profound influence on the results. Confirming this, biofilms grown under turbulent flow (e.g., using a CDC biofilm reactor) are less susceptible than when grown under laminar flow (e.g., using a DFBR) or a static (e.g., using a MTP method) biofilm system (Buckingham-Meyer et al. 2007; Nailis et al. 2010). Furthermore, the growth atmosphere can significantly affect the anti-biofilm activity. In this regard, CF *P. aeruginosa* biofilm grown under anaerobic conditions has shown to be more susceptible to colistin (Pompilio et al. 2015), and more resistant to tobramycin in an artificial sputum medium consisting of DNA from fish sperm, mucin from the porcine stomach, essential and non-essential L-amino acids, diethylenetriaminepentaacetic acid, NaCl, and KCl (Kirchner et al. 2012).

Models are often chosen based on their simplicity of use, their ability to reflect the growth and environmental survival conditions of the bacterial species tested, the preferences of the investigators, and the resources available.

However, a reliable *in vitro* model should take into consideration certain physiochemical and biological key elements to make the tests relevant for the intended clinical application to be considered (Table 3):

(i) biofilm should be grown under environmental conditions that resemble those observed at the infection site:
   - surface (in chronic infections, most biofilms form small aggregates of cells not attached to a surface, but embedded in host material) (Bjarnsholt et al. 2013); growth medium (e.g., artificial sputum medium, saliva, urine, blood) (Hill et al. 2010; Brackman and Coenye 2016; Pompilio et al. 2017; Kirchner et al. 2012); shear stress; pH; temperature; O_2 level (hypoxia until anaerobic niches were observed both in the wound and CF lung) (Sønderholm et al. 2017);
   - host immune response: knowledge of the interactions between biofilm bacteria and the immune system is critical to effectively address biofilm infections (Campoccia et al. 2019)

(ii) in the case of device-related infections, surface conditioning is needed when the device is expected to be exposed to a clinical environment prior to contacting microorganisms:
   - cardiovascular, as peripherally inserted central catheters, central venous catheters, and hemodialysis catheters (Rogers et al. 1996; Brooks and Keevil

Table 3 Criteria should be considered in choosing a reliable model for *in vitro* biofilm AST

Biofilm-related infection	*In vitro* models	Surface preconditioning[a]	Medium
Prostheses	CDC biofilm reactor	Urine (urinary catheteters)	Urine (urinary catheters)
	Robbins device	Blood components /fibrin, laminin, collagen), serum, plasma (CVC, hip prostheses)	Blood or serum (CVC[b], hip prostheses)
			Artificial saliva (ventilators)
Wound	Flow cell[c]		Collagen, fibronectin
	Duckworth biofilm model[d]		Serum, plasma or whole blood; saline
	Drip flow biofilm reactor		Simulated wound fluid
	Polaxamers		
	Alginate bead system		
	3D collagen model		
Cystic fibrosis lung	Flow cell	No surface	Artificial sputum medium
	Drip flow biofilm reactor		
	Polaxamers		
	Alginate beads		

[a]Preconditioning of the device is needed when it is expected to be exposed to a clinical environment prior to contacting microbes
[b]CVC, central venous catheter
[c]Flow cell allows for "Gram-negative shift"
[d]Duckworth model might be used as the test dressing can be applied directly on top of the biofilm, akin to the treatment of a real wound

1997; Al Akhrass et al. 2011): human or fetal bovine serum, plasma;
- respiratory, as endotracheal tubes, and ventilators (Leung and Darvell 1997): saline or artificial saliva;
- wound-related (e.g., wound dressings) (Hill et al. 2010; Poor et al. 2014): human or fetal bovine serum, plasma, whole blood, saline or water;

(iii) application of a dynamic, rather than static, environment to simulate the flow of biological fluids.

Furthermore, the method should allow for minimal sample handling to reduce contamination or to avoid alteration of biofilm structure during the testing.

8 Concluding Remarks

Recalcitrant and persistent biofilm-associated diseases require new, dedicated, therapeutic approaches. In this picture, *in vitro* methods for reliably culturing biofilms and evaluating their susceptibility to antibiotics are crucial in the assessment of therapeutic effectiveness. The choice of a biofilm model able to provide clinically relevant information is dependent upon several aspects in the *in vitro* design: selection of a suitable platform for generating biofilms with structural and physiological features resembling those observed at the site of infection, selection of physicochemical conditions mimicking the "environment" where the antibiotic will have to grapple with biofilm, and finally the use of clinically appropriate endpoints.

The complexity of this scenario makes the establishment of robust biofilm AST assays still a challenge for clinical entities. Furthermore, consideration and experimentation are, thus, warranted to:

- optimize or develop *in vitro* models where the conditions observed *in vivo* are accurately reproduced, also considering their

compatibility with routine clinical microbiology laboratory practice;
- standardize the procedures, parameters and breakpoints (MBIC, MBEC, BBC or BPC) to translate the obtained data to the clinical setting, including the comparative evaluation of different treatment strategies;
- perform *in vitro* studies comparing biofilm AST-driven therapy to conventional AST-driven therapy in the treatment of biofilm-related infections;
- carry out *in vivo* studies and clinical trials based on biofilm AST-driven therapy.

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Antibiofilm Efficacy of Polihexanide, Octenidine and Sodium Hypochlorite/Hypochlorous Acid Based Wound Irrigation Solutions against *Staphylococcus aureus, Pseudomonas aeruginosa* and a Multispecies Biofilm

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Abstract

Infection and the formation of biofilms have been shown to have a significant role in increased inflammation and delayed wound healing. Wound irrigation solutions are used to debride wounds, removing cell debris and infecting microorganisms, therefore preventing infection. The aim of this study was to evaluate a Polihexanide (PHMB) based wound irrigation solution, Octenidine HCl based wound irrigation solution and electrolysed water based wound care solution for antibiofilm efficacy against *Staphylococcus aureus, Pseudomonas aeruginosa* and a multispecies biofilm in several models to gain a broad understanding of ability. The PHMB based wound irrigation solution demonstrated broad range antibiofilm efficacy against *P. aeruginosa*, *S. aureus* and the multispecies biofilm. The Octenidine HCl based wound irrigation solution and the electrolysed water based wound care solution demonstrated potent antibiofilm efficacy against *S. aureus* and to a lesser extent *P. aeruginosa*. Overall, less efficacy was observed in the drip flow bioreactor model for all 3 test solutions, which may be attributed to the continuous flow of nutrients during treatment, which may have diluted or washed away the solution. The data presented also highlights the importance of testing antibiofilm activity in a range of biofilm models and against different bacterial strains to get an overall representation of efficacy.

Keywords

Antimicrobial · Biofilms · Multispecies · Polihexanide · Wound irrigation

1 Introduction

Multiple factors relating to a patient's underlying physiology are thought to contribute to delayed wound healing, including age, sex hormones, stress, diabetes and nutrition (Guo and Dipietro

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2010). Infection and the formation of biofilms in wounds has also been shown to have a significant role in increased inflammation and delayed wound healing (Banu et al. 2015; Percival 2017; Malone et al. 2017). Wound biofilms are formed when microbial cells adhere to a surface and each other and secrete extracellular polymeric substances (EPS), encasing themselves in a matrix (Flemming 2016). Biofilms are difficult to treat as once formed they are up to 1000x more tolerant to antimicrobials than their planktonic counterparts (Fleming et al. 2017).

Staphylococcus aureus and *Pseudomonas aeruginosa* are the most common microorganisms isolated from chronic wounds (Serra et al. 2015). Additionally, *S. aureus* and *P. aeruginosa* are both on the list of ESKAPE pathogens, the most common multidrug resistant (MDR) bacteria causing nosocomial infections (Esposito and De Simone 2017; Santajit and Indrawattana 2016). The ability of *S. aureus* and *P. aeruginosa* to form biofilms is also well documented (De Oliveira et al. 2016; Billings et al. 2013; Stewart et al. 2015). Although biofilms can be single species, they are often multispecies (O'Mar et al. 2017). Formation of multispecies biofilms has the potential to create a reservoir of antimicrobial resistance genes and an environment for genetic exchange, potentially contributing to MDR infections (Savage et al. 2013; Aguila-Arcos et al. 2017; Balcazar et al. 2015; Molin and Tolker-Nielsen 2003; Madsen et al. 2012; Stalder and Top 2016); therefore, demonstration of antibiofilm efficacy of an antimicrobial against *P. aeruginosa* and *S. aureus* as well as multispecies biofilms is important for a positive clinical outcome.

While there is currently no defined standard, representative of clinical treatment of a wound *in vitro*, there are standardised models that can be utilised for evaluating antibiofilm efficacy of wound care products. The minimum bactericidal eradication concentration (MBEC) model (ASTM E2799-17) involves growing the biofilm on a peg lid, under batch conditions, in a 96 well plate. The MBEC method is high throughput and allows simultaneous testing of multiple concentrations of the same test solution. The Centre for Disease Control (CDC) bioreactor model (ASTM E2871-19) allows growth of a biofilm in a vessel under constantly stirred conditions. The biofilm is grown under high shear conditions, which provides a more challenging biofilm to kill. The drip flow bioreactor model (ASTM E2647-20) allows growth of a biofilm close to the air/liquid interface under low shear conditions. Due to the continuous flow of proteinaceous media this model is more representative of an exuding wound environment and provides a further challenge to the efficacy of wound care products. The multispecies biofilm model involves growing the biofilm on filter discs using a hydrogel as a nutrient source to provide a dry environment for biofilm growth. Multispecies biofilms are often more challenging to eradicate, as different species in a biofilm often work synergistically together and can also provide an environment for genetic exchange of antimicrobial resistant genes (O'Mar et al. 2017; Aguila-Arcos et al. 2017). The LabTek chamber slide model involves growing the biofilm under batch conditions similar to the MBEC method; however, allows for a qualitative evaluation rather than quantitative. Qualitative evaluation is useful as it provides information on extracellular polymeric substance (EPS) breakdown/biofilm disruption and removal that may not be identified from quantitative evaluation.

Wound irrigation solutions are used to cleanse, rinse and moisturise wounds, effectively debriding wounds of cell debris, slough, eschar and microorganisms and therefore reducing wound healing time and preventing biofilm formation and infection. Addition of antimicrobial agents and surfactants in wound irrigation solutions has been shown to enhance wound healing in comparison to normal saline solution (Percival et al. 2017; Bellingeri et al. 2016). The aim of this study was to evaluate the antibiofilm efficacy of a 0.1% Polihexanide (PHMB) based wound irrigation solution (Prontosan®, B Braun Medical), Octenidine HCl based wound irrigation solution (Octenilin®, Schülke & Mayr GmbH) and electrolysed water (sodium hypochlorite and hypochlorous acid) based wound care solution (Microdacyn 60®, Sonoma™ Pharmaceuticals)

in multiple *in vitro* models to gain a broad understanding of their antibiofilm capability.

2 Materials and Methods

2.1 Test Articles

A 0.1% Polihexanide (PHMB) based wound irrigation solution containing 0.1% Betaine and purified water (Prontosan®, B Braun Medical), Octenidine HCl based wound irrigation solution containing aqua valde purificata, Glycerin and Ethylhexylglycerin (Octenilin®, Schülke & Mayr GmbH) and electrolysed water (sodium hypochlorite and hypochlorous acid) based wound care solution containing super-oxidized water and 0.022% sodium chloride (Microdacyn 60®, Sonoma™ Pharmaceuticals).

A broad-spectrum neutraliser consisting of 30 g/L Tween 80, 3 g/L Lecithin, 1 g/L L-Histidine, 2 g/L L-Cysteine and 15 g/L Saponin was used to neutralise all of the wound irrigation solutions throughout this study. All reagents were purchased from Sigma-Aldrich, UK.

2.2 LabTek Chamber Slide Biofilm Model

The antibiofilm efficacy of the test solutions against *P. aeruginosa* ATCC 15442 and *S. aureus* ATCC 29213 was evaluated qualitatively by growing the biofilms in LabTek chamber slides and staining them with LIVE/DEAD™ *Bac*Light™ bacterial viability kit (ThermoFisher Scientific, UK). The biofilms were then treated with each solution for 24 h before visualising them using confocal laser scanning microscopy (CLSM).

Overnight cultures of *P. aeruginosa* ATCC 15442 and *S. aureus* ATCC 29213 were set up by inoculating Tryptone Soya broth (TSB) with a single colony and incubating it overnight at 37 °C and 125 rpm. The following day, the overnight cultures were adjusted to 1×10^6 CFU/mL and added to LabTek chamber slides. The slides were incubated at 37 °C and 125 rpm for 24 h.

Following growth, the biofilm was washed twice with phosphate buffered saline (PBS) and stained with the LIVE/DEAD™ *Bac*Light™ kit (ThermoFisher Scientific, UK) by preparing a 2× solution of SYTO 9 and propidium iodide fluorescent stains and adding it to the LabTek chamber slides. The slides were incubated in the dark for 15 min to allow staining of the cells, before washing the biofilms twice with PBS and adding each test solution at 100% concentration in triplicate. PBS only was added to the untreated control and the slides were incubated for 24 h.

Following incubation, each well was visualised using an LSM 780 imaging confocal microscope and Zeiss software at an excitation/emission of 480/500 nm. For each chamber, 16 images were taken and collated into 1 image to show the entire surface of each chamber. Images were processed using Image J software (National Institutes of Health, USA).

2.3 Minimum Biofilm Eradication Concentration (MBEC) Model

The antibiofilm efficacy of the 3 wound solutions was evaluated against a 24 h biofilm of *P. aeruginosa* ATCC 15442 and *S. aureus* ATCC 29213 following an adapted version of ASTM standard E2799-17 'Standard Test Method for Testing Disinfectant Efficacy against *Pseudomonas aeruginosa* Biofilm using the MBEC Assay.'

Briefly, overnight cultures of *P. aeruginosa* ATCC 15442 and *S. aureus* ATCC 29213 were set up by inoculating TSB with a single colony and incubating at 37 °C and 125 rpm in an orbital shaking incubator. The following day the overnight cultures were adjusted to 1×10^5 CFU/mL in TSB and used to inoculate a 96 well plate. A peg lid was placed onto the 96 well plate which was incubated at 37 °C and 110 rpm for 24 h.

The challenge plates for each test solution were set up by adding the solution to a new 96 well plate and serial diluting it 1:2 in PBS. A neutraliser effectiveness control was set up by adding each solution to the neutraliser at a ratio of 1:1. A neutraliser toxicity control was set up by

adding the neutraliser only and an untreated control was set up by adding PBS only to the plate.

The peg lid containing biofilm was transferred to a rinse plate containing PBS before transferring it to the challenge plate. The challenge plates containing *P. aeruginosa* were incubated at room temperature for 24 h. The challenge plates containing *S. aureus* were incubated at 37 °C for 24 h.

Following 24 h treatment, the peg lids were transferred to recovery plates containing neutraliser and sonicated on full power (100 W) in an Ultrawave water bath for 30 min. Samples were transferred to new 96 well plates and serial diluted 1:10. Serial dilutions were spot plated onto Tryptone Soya agar (TSA) and incubated overnight at 37 °C. The following day counts were enumerated.

2.4 Centers for Disease Control (CDC) Bioreactor Model

The antibiofilm efficacy of the 3 wound solutions was also evaluated in the CDC bioreactor model against 24 h biofilms of *P. aeruginosa* ATCC 15442 and *S. aureus* ATCC 29213.

Overnight cultures of *P. aeruginosa* ATCC 15442 and *S. aureus* ATCC 29213 were set up by inoculating TSB with a single colony and incubating at 37 °C and 125 rpm. The overnight culture of *P. aeruginosa* was adjusted to 1×10^8 CFU/mL in TSB and used to inoculate the CDC bioreactor. The CDC bioreactor was then incubated at room temperature and 125 rpm for 24 h. The overnight culture of *S. aureus* was pelleted by centrifugation, resuspended in TSB and used to inoculate the CDC bioreactor. The CDC bioreactor was incubated for 24 h on at 37 °C and 100 rpm.

Following 24 h incubation, coupons were washed twice in PBS and placed into 6 well plates. Each neat wound solution was added to wells in triplicate (4 mL/well). A set of untreated coupons were also added to the wells for a comparison growth control. The plates were then incubated for 24 h at room temperature.

The following day, coupons were removed from wells, added to neutraliser and sonicated on full power for 30 min. Samples were vortexed briefly, serial diluted 1:10 in PBS and plated onto TSA. Plates were incubated at 37 °C overnight and the following day colony counts were enumerated.

2.5 Drip Flow Bioreactor Model

The antibiofilm efficacy of the test solutions was evaluated in the drip flow bioreactor model following ASTM E2647-13 Quantification *of Pseudomonas aeruginosa* Biofilm Grown Using Drip Flow Biofilm Reactor with Low Shear and Continuous Flow.

The drip flow bioreactor was prepared by adding a clean borosilicate microscope slide to each channel and autoclaving at 121 °C for 15 min. An overnight inoculum was set up by inoculating TSB with a single colony of *P. aeruginosa* ATCC 700888 and incubating at 37 °C and 125 rpm. The following day, the overnight culture was adjusted to 1×10^8 CFU/mL and used to inoculate the drip flow bioreactor chambers. The drip flow bioreactor was incubated for 6 h in batch phase before connecting it to a nutrient carboy containing 270 mg/L TSB operated continuously at a flow rate of 50 mL/h/channel.

After 24 h biofilm growth, sterile gauze was soaked in the test solutions for 30 min and added to microscope slides in triplicate. The biofilms were incubated in continuous phase for a further 24 h. After the challenge period, each microscope slide was scraped into 45 mL of neutraliser washed with 5 mL neutraliser. Each sample was homogenised for 30 s before serial diluting it 1:10 in PBS and plating it out in duplicate onto TSA. The plates were incubated overnight at 37 °C and the following day counts were enumerated.

2.6 Multispecies Biofilm Model

The antibiofilm efficacy of the 3 wound solutions was evaluated against a 24 h multispecies biofilm of *P. aeruginosa* ATCC 15442, *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212. The biofilm was grown on filter discs using a hydrogel as a nutrient source.

The hydrogel was prepared by dissolving 3-sulfopropyl acrylate potassium salt (polymer) in PBS and then adding PEG dissolved in PBS, foetal bovine serum (FBS) and 1% 1-hydroxy cyclohexyl phenol ketone prepared in 70% ethanol (photo-initiator) to it. The mixture was added to a 12 well plate (2 mL/well) and set by exposing the hydrogel to 366 nm UV light.

Overnight cultures of *P. aeruginosa* ATCC 15442, *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 were set up by inoculating TSB with a single colony and incubating at 37 °C and 125 rpm. Overnight cultures were adjusted to 1×10^8 CFU/mL before adding all 3 strains together in TSB at a final concentration of 1×10^6 CFU/mL. Durapore 13 mm (1 μM) membrane filter discs (Merck, UK) were incubated with the culture for 2 h at 37 °C and 125 rpm. Following this, the filters were transferred to a 12 well plate containing the hydrogel and incubated at 37 °C for 24 h.

Following 24 h biofilm growth, the filter discs were transferred to fresh 12 well plates and treated with each solution in triplicate. PBS was added to the untreated control in triplicate. Biofilms were treated for 24 h at 37 °C. Following 24 h treatment, filter discs were transferred to neutraliser and sonicated on full power for 30 min. Samples were vortexed briefly, serial diluted 1:10 in PBS and plated onto TSA. The plates were incubated overnight at 37 °C and the following day counts were enumerated.

2.7 Statistical Analysis

Raw data was entered into Microsoft Excel and average CFU/mL was calculated. To determine if there was a statistical difference between the untreated control and the treated biofilms one-way ANOVA using Dunnett's multiple comparison test was carried out using Prism 7 software.

3 Results

3.1 Antibiofilm Efficacy in the LabTek Chamber Slide Model

The antibiofilm efficacy of the test solutions was evaluated qualitatively against 24 h biofilms of *S. aureus* ATCC 29213 and *P. aeruginosa* ATCC 15442.

All 3 test solutions demonstrated efficacy against *P. aeruginosa* (Fig. 1) and *S. aureus* (Fig. 2) biofilms, with evidence of biofilm disruption and removal of the biofilms being observed in all the treated wells in comparison to the untreated control.

3.2 Antibiofilm Efficacy in the MBEC Model

Following treatment of a 24 h *P. aeruginosa* biofilm and *S. aureus* biofilm with the PHMB based wound irrigation solution at 100% concentration, complete eradication of the biofilms were found (Fig. 3). In comparison the untreated growth controls had a bacterial cell density of 2.84×10^4 CFU/mm^2 ($p < 0.0001$) and 7.29×10^4 CFU/mm^2 (p 0.0003), respectively showing a 4 log reduction in both biofilms. The PHMB based wound irrigation solution also demonstrated efficacy against the *P. aeruginosa* biofilm at 50%, 25% and 12.5% concentration, showing a 2–4 log reduction in bacterial cell density ($p \leq 0.0308$) and at 50%, 25%, 12.5% and 6.25% concentration against the *S. aureus* biofilm, showing a 1.5–3 log reduction in bacterial cell density ($p \leq 0.0321$).

Following treatment with the Octenidine HCl based wound irrigation solution at 100%, 50%, 25%, 12.5%, 6.25% and 3.13% antibiofilm efficacy was observed against a 24 h biofilm of

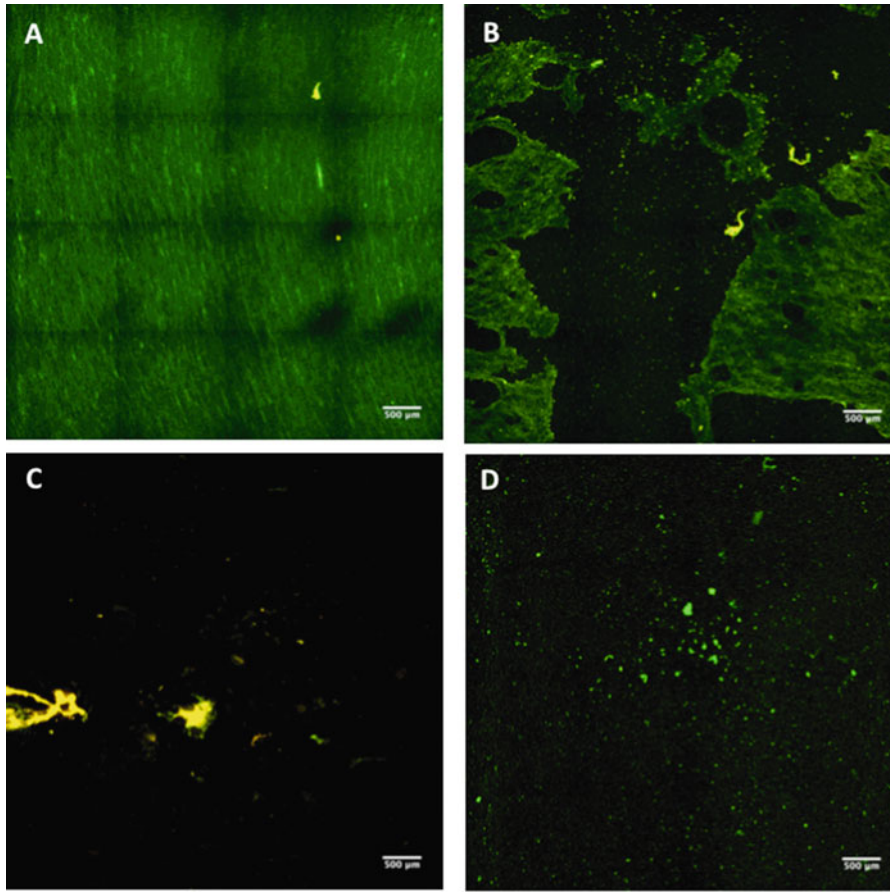

Fig. 1 Representative images of *P. aeruginosa* biofilm on the surface of the Lab Tek chamber slide. Biofilms were stained with LIVE/DEAD BacLight stain. The images show the untreated biofilm (**a**) and biofilm following treatment with the PHMB wound irrigation solution (**b**), the Octenidine HCl based wound irrigation solution (**c**) and the electrolysed water based wound care solution (**d**)

P. aeruginosa, with a bacterial cell density ranging from 5.83 x 10^3 to 1.36 $\times$ 10^4 CFU/mm^2. In comparison the untreated growth control had a bacterial cell density of 1.66 $\times$ 10^5 CFU/mm^2 showing a 1–1.5 log reduction in biofilm ($p \leq 0.0376$). Following treatment with the Octenidine HCl based wound irrigation solution at 100% and 50% complete eradication of a 24 h *S. aureus* biofilm was found. In comparison the untreated biofilm growth control had a bacterial cell density of 2.90 $\times$ 10^5 CFU/mm^2 showing a 5 log reduction ($p < 0.0001$). At 25%, 12.5% and 6.25% concentration, the Octenidine HCl based wound irrigation solution also demonstrated antibiofilm efficacy showing a 2–5 log reduction in bacterial cell density ($p \leq 0.0423$).

Following treatment of a 24 h *P. aeruginosa* biofilm with the electrolysed water based wound care solution at 100% and 50% concentration, complete eradication of the biofilm was found. In comparison the untreated biofilm growth control had a bacterial cell density of 4.02 $\times$ 10^4 CFU/mm^2 showing a 4 log reduction following treatment (p 0.0005). The electrolysed water based wound care solution at 25% and 12.5% concentration also showed antibiofilm efficacy with a 3–4 log reduction ($p \leq 0.0221$). Following treatment of a 24 h *S. aureus* biofilm with the electrolysed water based wound care solution

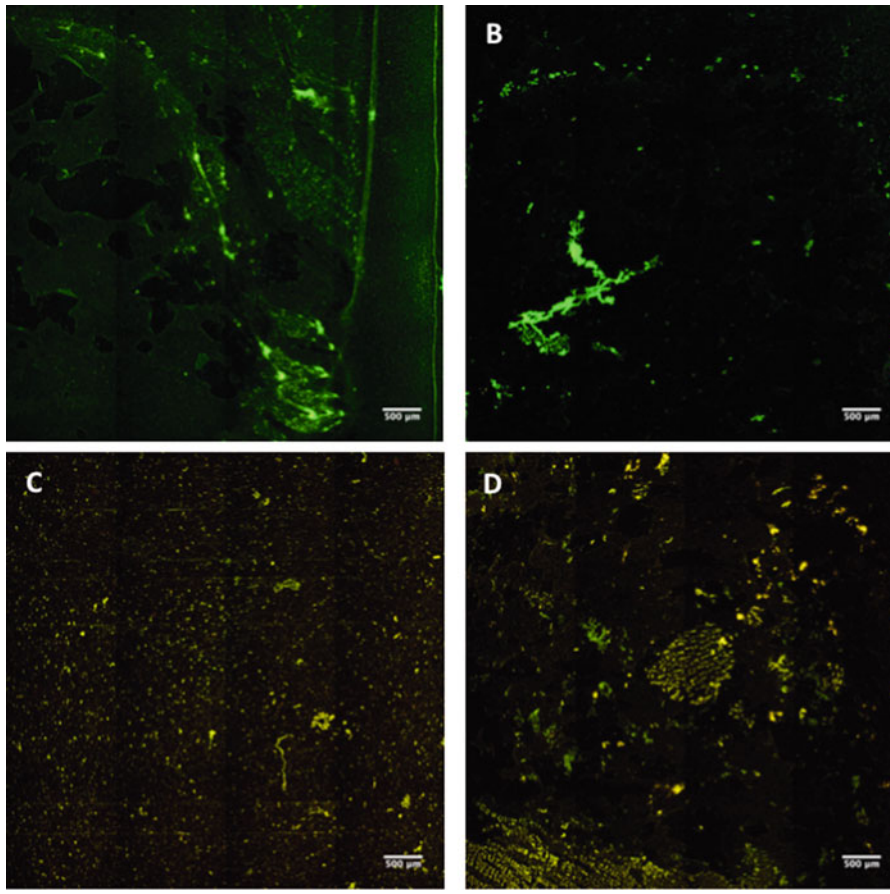

Fig. 2 Representative images of *S. aureus* biofilm on the surface of the Lab Tek chamber slide. Biofilms were stained with LIVE/DEAD BacLight stain. The images show the untreated biofilm (**a**) and biofilm following treatment with (**b**), the Octenidine HCl based wound irrigation solution (**c**) and the electrolysed water based wound care solution (**d**)

at 100% concentration, complete eradication of the biofilm was found. In comparison the untreated biofilm growth control had a bacterial cell density of 1.98×10^5 CFU/mm^2 showing a 5 log reduction ($p < 0.0001$). The electrolysed water based wound care solution at 50% and 25% concentration also showed antibiofilm efficacy against *S. aureus*, with a 1 log reduction being found following treatment ($p \leq 0.0058$).

The neutraliser effectiveness and neutraliser toxicity controls in this model demonstrated that it neutralised all 3 of the wound irrigation solutions and that it was also non-toxic to the bacteria.

3.3 Antibiofilm Efficacy in the CDC Bioreactor Model

The antibiofilm efficacy of the 3 test solutions was also evaluated in the CDC bioreactor model and all showed antibiofilm efficacy against 24 h biofilms of *P. aeruginosa* ATCC 15442 (Fig. 4) and *S. aureus* ATCC 29213 (Fig. 5) to different extents.

Following 24 h growth of *P. aeruginosa* ATCC 15442 biofilm in the CDC bioreactor model, the untreated growth control had a bacterial cell density of 4.53×10^7 CFU/mL. The biofilm treated with the PHMB based solution,

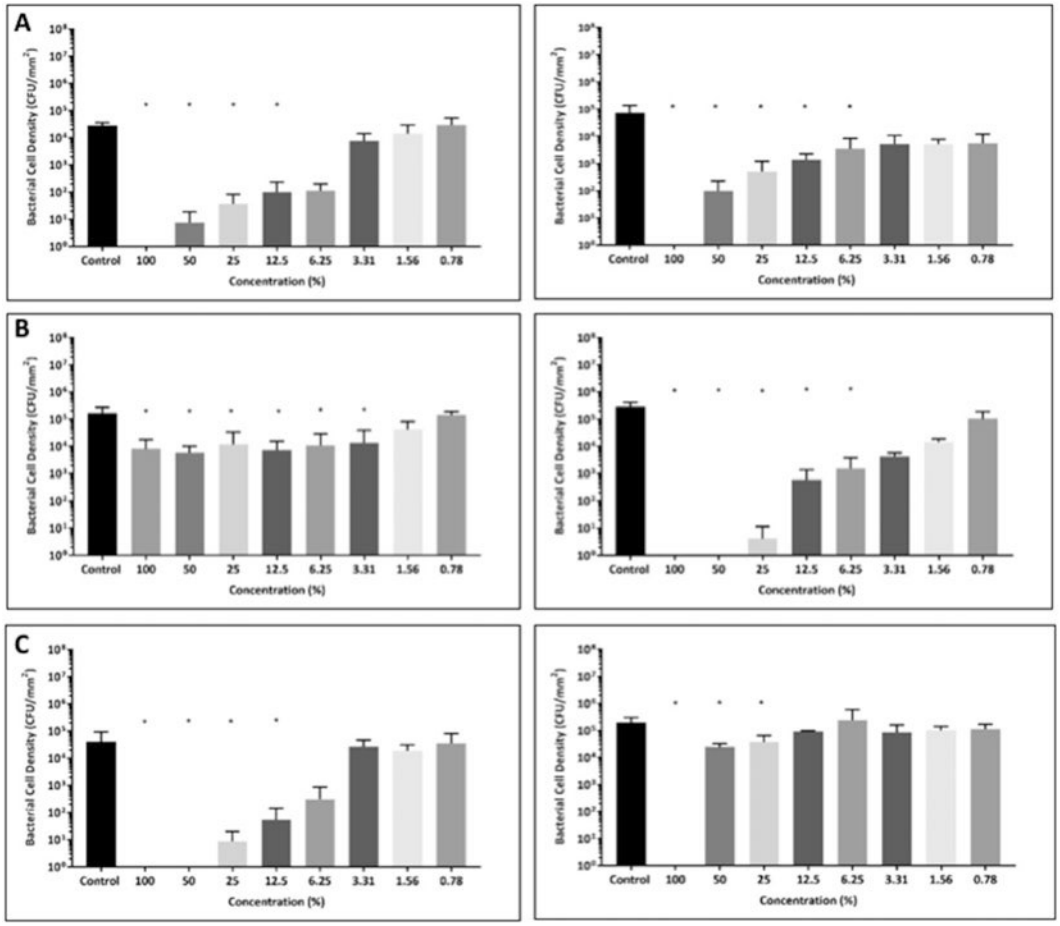

Fig. 3 MBEC of the PHMB based wound irrigation solution (**a**), the Octenidine HCl based wound irrigation solution (**b**) and the electrolysed water based wound care solution (**c**) against a 24 h biofilm of *P. aeruginosa* ATCC 15442 (left) and *S. aureus* ATCC 29213 (right). Error bars represent the standard error of the mean. * = a statistically significant reduction in biofilm in comparison to the untreated control ($p \leq 0.0423$)

Octenillin solution and electrolysed water solution had a bacteria cell density of 3.00 x 10^2 CFU/mL, 2.80 $\times$ 10^4 CFU/mL and 3.33 $\times$ 10^6 CFU/mL, showing a 5 (p 0.0005), 3 (p 0.0005) and 1 (p 0.0009) log reduction in biofilm compared to the untreated control, respectively.

Following 24 h treatment of the *S. aureus* ATCC 29213 biofilm, the growth control had a bacterial cell density of 4.93 $\times$ 10^6 CFU/mL. Treatment with each solution resulted in complete eradication of the biofilm, showing a 6 log reduction in bacterial cell density in comparison to the untreated control (p 0.0001).

3.4 Antibiofilm Efficacy in the Drip Flow Bioreactor Model

The antibiofilm efficacy of the test solutions was evaluated against a 24 h biofilm of *P. aeruginosa* ATCC 700888 in the drip flow bioreactor model, by growing the biofilm and applying the treatment with a continuous supply of nutrients (Fig. 6).

Following 24 h treatment of the *P. aeruginosa* biofilm with the PHMB based wound irrigation solution, a bacterial cell density of 2.54 $\times$ 10^6 CFU/mL was found. In comparison

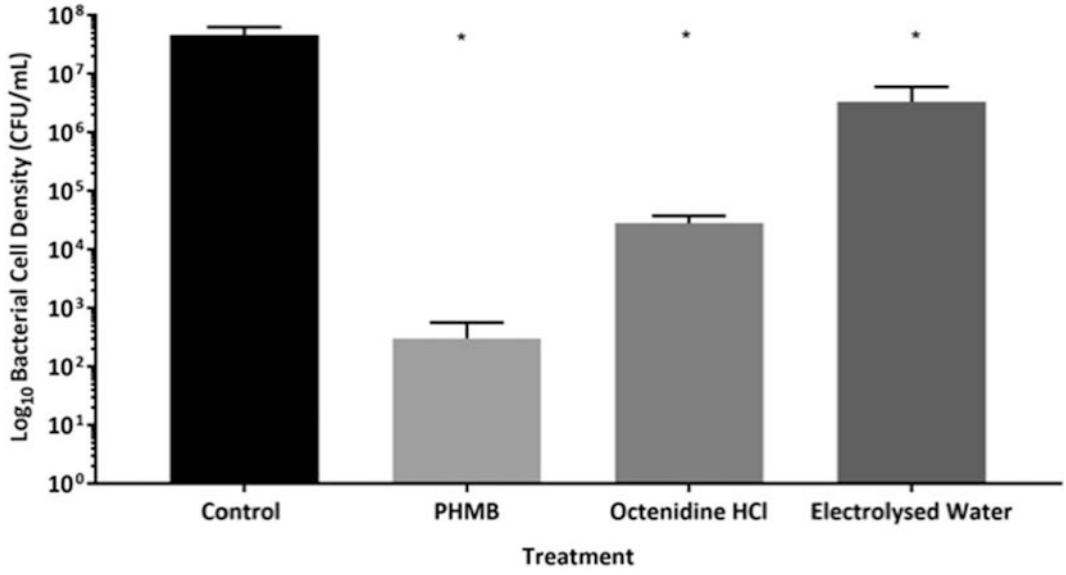

Fig. 4 Log_{10} bacterial cell density of *P. aeruginosa* ATCC 15442 24 h biofilm following treatment with the PHMB based wound irrigation solution, the Octenidine HCl based wound irrigation solution and the electrolysed water based wound care solution. Error bars represent the standard error of the mean. * = a statistically significant reduction in biofilm in comparison to the untreated control ($p \leq 0.0009$)

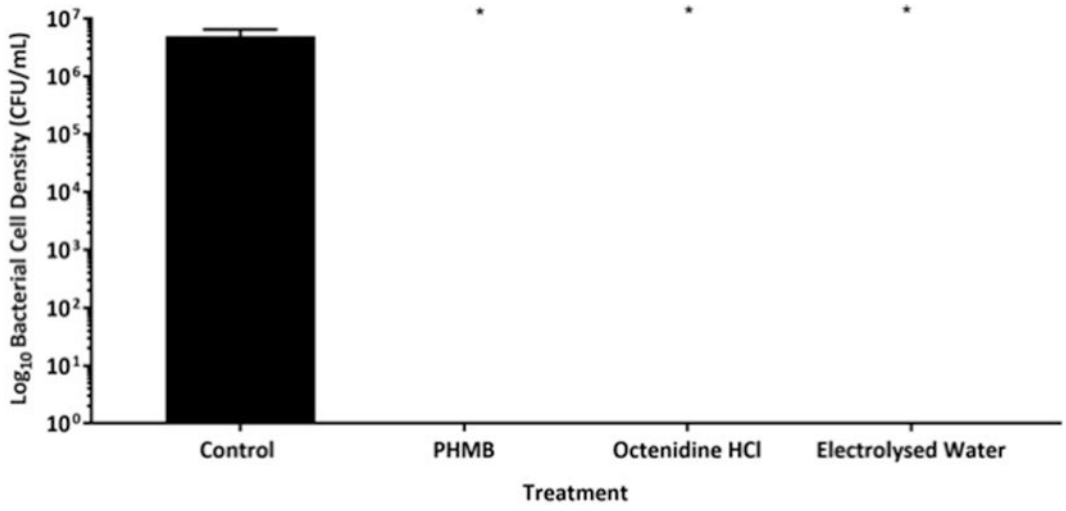

Fig. 5 Log_{10} bacterial cell density of *S. aureus* ATCC 29213 24 h biofilm following treatment with the PHMB based wound irrigation solution, the Octenidine HCl based wound irrigation solution and the electrolysed water based wound care solution. Error bars represent the standard error of the mean. * = a statistically significant reduction in biofilm in comparison to the untreated control (p 0.0001)

the untreated biofilm had a bacterial cell density of 2.15×10^9 CFU/mL, showing a 3 log reduction. Treatment with the Octenidine HCl based wound irrigation solution and the electrolysed water based wound care solution resulted in a bacterial cell density of 5.40×10^5 CFU/mL

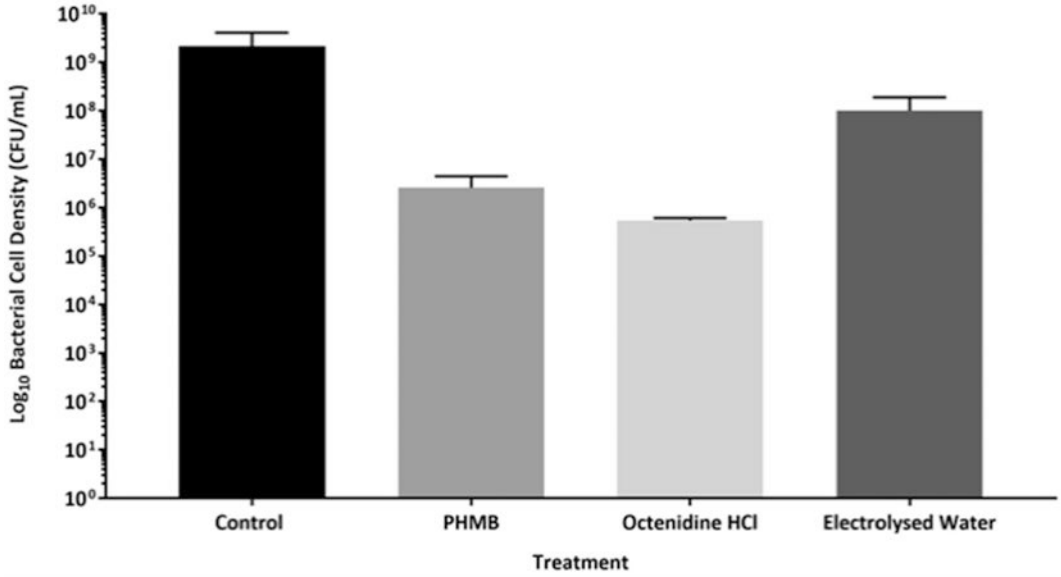

Fig. 6 Log_{10} bacterial cell density of *P. aeruginosa* ATCC 700888 24 h biofilm following treatment with the PHMB based wound irrigation solution, the Octenidine HCl based wound irrigation solution and the electrolysed water based wound care solution. Error bars represent the standard error of the mean

and 9.93×10^7 CFU/mL, showing a 3.5 log and 1.5 log reduction in comparison to the untreated control, respectively.

3.5 Antibiofilm Efficacy in the Multispecies Biofilm Model

The antibiofilm efficacy of the test solutions was evaluated against a 24 h multispecies biofilm of *P. aeruginosa*, *S. aureus* and *E. faecalis* by growing the biofilm on filter discs with a hydrogel as a nutrient supply.

Following 24 h treatment with the PHMB based wound irrigation solution and the electrolysed water based wound care solution no colonies were observed, showing complete eradication of the biofilm (p 0.0016; Fig. 7). In comparison the untreated biofilm had a bacterial cell density of 1.07×10^6 CFU/mL showing a 6 log reduction with the treatments. Following treatment with the Octenidine HCl based wound irrigation solution a bacterial cell density of 3.10×10^3 CFU/mL was found, showing a 3 log reduction in biofilm in comparison to the untreated control (p 0.0016).

4 Discussion

In this study, the antibiofilm efficacy of a PHMB based wound irrigation solution in comparison to an Octenidine HCl based wound irrigation solution and the electrolysed water based wound care solution was evaluated in several different models against *P. aeruginosa*, *S. aureus* and a multispecies biofilm of *P. aeruginosa*, *S. aureus* and *E. faecalis*. The solutions were applied at 100% concentration and at diluted concentrations for a contact time of 24 h to represent clinical application of wound irrigation solutions when they are used longer term in comparison to short contact times such at 15 min.

The PHMB based wound irrigation solution contains the PHMB as an antimicrobial preservative and the surfactant Betaine. PHMB has been shown to have a broad spectrum of activity against bacteria, yeast and fungi through cell

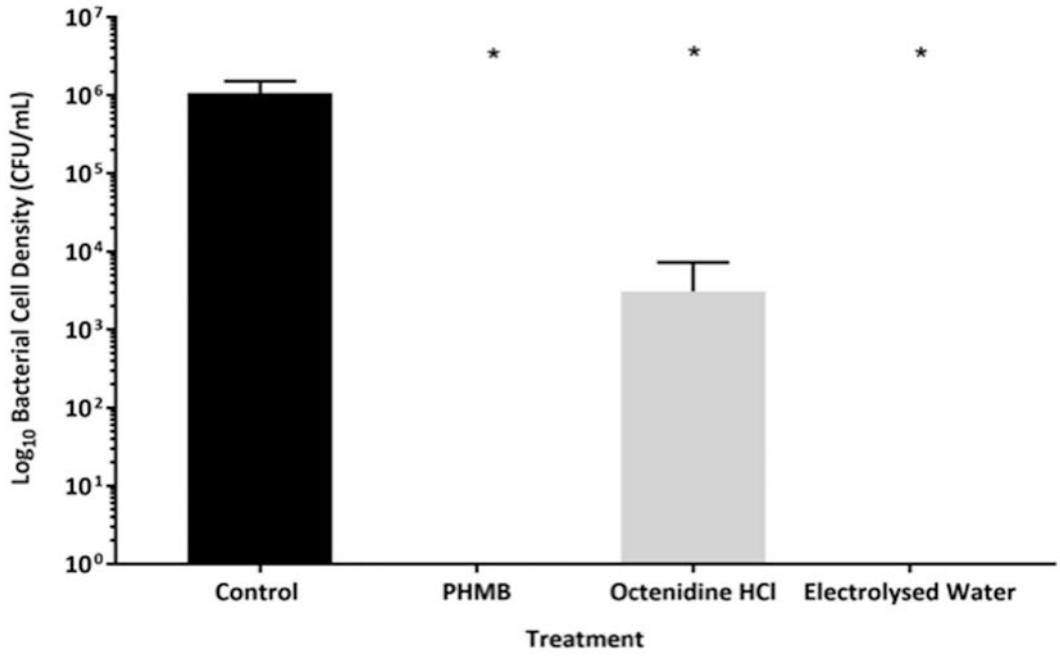

Fig. 7 Log_{10} bacterial cell density of a 24 h multispecies biofilm following treatment with the PHMB based wound irrigation solution, the Octenidine HCl based wound irrigation solution and the electrolysed water based wound care solution. Error bars represent the standard error of the mean. * = a statistically significant reduction in biofilm in comparison to the untreated control (p 0.0016)

membrane disruption; although the ability to selectively enter bacterial cells and bind chromosomes has also been shown (Ali and Wilson 2017; Chindera et al. 2016; Kamaruzzaman et al. 2016; Rembe et al. 2016). Treatment of wounds with PHMB has been associated with a positive clinical outcome (Webster et al. 2017; To et al. 2016; Villela-Castro et al. 2018). Surfactants, such as Betaine, are widely used in wound care to aid cleaning and debridement of wounds (Percival et al. 2017; Bellingeri et al. 2016). The PHMB based wound irrigation solution has shown the ability to break down dried human plasma, representative of wound coatings *in vitro* and also clean, moisten and decontaminate encrusted chronic wounds in a small clinical study of 10 patients (Horrocks 2006; Kaehn and Eberlein 2009). In a previous study, the PHMB based wound irrigation solution demonstrated antimicrobial activity against *S. aureus* (Hirsch et al. 2011). Additionally, treatment of chronic wounds, such as venous leg ulcers, with the PHMB based wound irrigation solution has been associated with an improved clinical outcome, with wounds healing faster and more wounds completely healing (Andriessen and Eberlein 2008).

Octenidine has demonstrated broad spectrum antimicrobial activity *in vitro* (Alvarez-Marin et al. 2017; Assadian 2016). Additionally, following central venous catheter insertion in a double-blind randomized controlled trial, the Octenidine HCl based wound irrigation solution significantly reduced insertion site skin colonisation and colonisation of the catheter tip (Dettenkofer et al. 2010). The Octenidine HCl based wound irrigation solution has also been shown to effectively reduce MRSA colonisation in observational studies (Krishna and Gibb 2010). The electrolysed water based wound care solution contains 0.004% sodium hypochlorite and 0.004% hypochlorous acid as preservative agents. The electrolysed water based wound care solution primarily debrides wounds, decreasing infection

rates and improving wound healing. A randomised single-blind clinical control study of patients with diabetic foot ulcers demonstrated the electrolysed water based wound care solution to be more efficacious in infection control, odour reduction and erythema reduction than conventional disinfectants (Martinez-De Jesus et al. 2007). However, some studies have demonstrated that sodium hypochlorite/hypochlorous acid wound care solutions with low total chlorine have low antimicrobial and antibiofilm efficacy (Severing et al. 2019; Krasowski et al. 2021).

In this study, the antibiofilm efficacy of all 3 solutions was evaluated in multiple models against *S. aureus* and *P. aeruginosa*, which are commonly associated with wound infections (Serra et al. 2015). The LabTek chamber slide model involves growing the biofilm in batch phase and staining the biofilm with SYTO 9 and propidium iodide fluorescent stains, to allow visualisation of the biofilm and qualitative analysis following treatment with test solutions. Using this method, disruption and removal of both *P. aeruginosa* and *S. aureus* biofilms could be observed following treatment with all 3 test solutions. It has been shown previously that in models such as the LabTek chamber slide model, pipette-based wash steps can cause random holes and alterations within the biofilms as it is quite an aggressive method (Tasse et al. 2018). To account for possible biofilm removal through use of the pipette washing technique, an untreated biofilm control that was subject to the same washing and staining steps was used as a comparison when visually analysing the effects of each treatment. Additionally, each group was tested in triplicate to allow consistent results to be drawn from each treatment group compared to the untreated group. Although these measures were taken to account for any biofilm disruption caused by the methodology rather than the treatment, the limitations of this model should be taken into consideration when reviewing the data.

The MBEC model involves growing the biofilm under batch conditions, so there is no flow of nutrients and allows high throughput testing so that multiple concentrations of test solution can be evaluated simultaneously. In this model, the PHMB based wound irrigation solution showed complete eradication of the *P. aeruginosa* and *S. aureus* biofilms at 100% concentration. Complete eradication of the *P. aeruginosa* biofilm was also found with the electrolysed water based wound care solution at 50% and complete eradication of the *S. aureus* biofilm was found with the Octenidine HCl based wound irrigation solution at 50% and the electrolysed water based wound care solution at 100%. The Octenidine HCl based wound irrigation solution was less efficacious in this model against *S. aureus* but showed some activity with a 1.5 log reduction in biofilm in comparison to the untreated control. The MBEC of the wound irrigation solutions has been evaluated elsewhere recently using a 96-well plate method and 1% tetrazolium chloride staining method (Krasowski et al. 2021). Krasowski et al. showed the PHMB based wound irrigation solution eradicated the *S. aureus* and *P. aeruginosa* biofilms at approx. 20% and 35% concentration, respectively, whilst the Octenidine solution eradicated them at approx. 10% and 40%, respectively. The electrolysed water based wound care solution did not eradicate the biofilm at up to 50% concentration. The differences in concentration required to fully eradicate the biofilms between the 2 studies may be as a result of differences in the methodology, such as the sensitivity of bacterial detection methods and that different bacterial strains used.

The CDC bioreactor model involves growing a biofilm under high shear conditions and adding the test samples in a dry environment to the biofilm containing coupons. In this model, the PHMB based wound irrigation solution showed the greatest antibiofilm efficacy against a *P. aeruginosa* biofilm, reducing it by 5 log in comparison to the untreated control. The Octenidine HCl based wound irrigation solution and the electrolysed water based wound care solution showed some antibiofilm activity with a 3 log and 1 log reduction, respectively. All 3 test solutions demonstrated greater efficacy against *S. aureus* in this model, with complete eradication of the biofilm being found following treatment.

The drip flow bioreactor model involves growing a *P. aeruginosa* biofilm close to the air/liquid

interface in an environment with continuous nutrient flow under low shear conditions. The nutrient flow is continued during treatment application and is designed to represent a highly exudative wound environment. In this model, the PHMB based wound irrigation solution and the Octenidine HCl based wound irrigation solution both showed antibiofilm efficacy, reducing the bacterial cell density by 3 log and 3.5 log, respectively. The electrolysed water based wound care solution was less efficacious, showing a 1 log reduction in the *P. aeruginosa* biofilm.

The test solutions were also evaluated against a multispecies biofilm model of *P. aeruginosa*, *S. aureus* and *E. faecalis*, as clinical studies have shown that biofilms are often multispecies rather than single species (Alexiou et al. 2017). Additionally, all 3 strains are commonly associated with nosocomial infections and wound biofilms (Krishna and Gibb 2010; Banu et al. 2015; Serra et al. 2015; Obermeier et al. 2018; Faron et al. 2016). Therefore, the biofilm in this model may be more representative of a wound environment. In this model, the PHMB based wound irrigation solution and the electrolysed water based wound care solution eradicated the multispecies biofilm showing potent antibiofilm efficacy. The Octenidine HCl based wound irrigation solution also demonstrated antibiofilm efficacy, with treatment resulting in a 3 log reduction of the biofilm.

Overall, the PHMB based wound irrigation solution demonstrated potent antibiofilm efficacy across most of the biofilm models used in this study, with treatment resulting in complete eradication of the biofilm and a 5 log reduction of a *P. aeruginosa* biofilm grown in the CDC bioreactor model. The Octenidine HCl based wound irrigation solution showed potent antibiofilm efficacy against *S. aureus*, completely eradicating the biofilm in both the MBEC and CDC bioreactor model. Although some antibiofilm activity was found against *P. aeruginosa* and the multispecies biofilm, the Octenidine HCl based wound irrigation solution was less efficacious against these biofilms than the *S. aureus* one. The electrolysed water based wound care solution completely eradicated the *S. aureus* biofilm in the MBEC and CDC bioreactor models and the multispecies biofilm, showing potent efficacy against these biofilms; however, less efficacy was observed against *P. aeruginosa* in the CDC bioreactor model and the drip flow bioreactor model. Overall, less efficacy was observed in the drip flow bioreactor model for all 3 test solutions, which may be attributed to the continuous flow of proteinaceous media during treatment, which may have diluted or washed away the solution.

The data presented in this study shows the PHMB based wound irrigation solution to have the greatest broad range antibiofilm activity against both *P. aeruginosa*, *S. aureus* and a multispecies biofilm in comparison to the other solutions tested. The Octenidine HCl based wound irrigation solution demonstrated potent antibiofilm activity against *S. aureus*, but to a lesser extent against *P. aeruginosa* and the multispecies biofilm and the electrolysed water based wound care solution demonstrated potent antibiofilm activity against *S. aureus* and the multispecies biofilm, but to a lesser extent against *P. aeruginosa*. The data presented also highlights the importance of testing antibiofilm activity in a range of biofilm models and against different bacterial strains to get an overall representation of efficacy.

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Molecular Characterization of Extended-Spectrum Beta-Lactamase-Producing Enterobacteriaceae Isolates Collected from Inanimate Hospital Environments in Addis Ababa, Ethiopia

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Abstract

Introduction The hospital environment contributes to the spread of Extended-spectrum β-lactamase-producing *Enterobacteriaceae* (ESBL-PE), which are contributing to increased morbidity and mortality rates. The present study was carried out to detect environmental contamination, antimicrobial susceptibility testing of ESBL-PE, and to explore molecular characterization of ESBL encoding genes.

Methods A cross-sectional study was conducted within the intensive care units (ICUs) of Tikur Anbessa Specialized Hospital from June to July 2018. A total of 97 swabs were taken from high-contact inanimate surfaces near immediate patient environments. All isolates were cultured by using ESBL ChromoSelect Agar and identified with conventional bacteriological methods. Antimicrobial susceptibility testing was performed as recommended by Clinical and Laboratory Standards Institute. Combination disk test was used to confirm ESBL production, while molecular characterizations of ESBL genes were performed by polymerase chain reaction.

Results Out of 97 swabbed sample, 24 (24.7%) were confirmed as ESBL-PE. The most predominant ESBL-PE was from *E. coli* (41.7%) and *K. pneumoniae* (25%). The

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Pediatrics and Neonatal ICU (29.2%, 7/24) exhibited highest ESBL-PE. The most contaminated materials were bed linens (33.3%). Most of ESBL-PE isolates were resistant to ampicillin (100%) and ceftriaxone (91.7%). A low resistance level was recorded for amikacin (25%). Among ESBL-producing genes, bla_{CTX-M} (35.7%) was the most prevalent, followed by bla_{TEM} and bla_{SHV} gene 32.1% for each.

Conclusions Appearance of ESBL-PE in ICUs environment is posing a serious threat to control healthcare associated infections. The high level of resistance shows the need of policies for devising infection control procedures and detection of ESBL-PE.

Keywords

Antimicrobial susceptibility · *Enterobacteriaceae* · ESBL · Inanimate hospital environment · Molecular characterization

Abbreviations

%	Percent
°C	Degree Celsius
ATCC	American Type Culture Collection
CD	combination disk
CLSI	Clinical Laboratory Standards Institute
DRERC	Department of Microbiology, Immunology and Parasitology Research Ethics Review Committee
ESBL-PE	Extended-spectrum β-lactamase producing *Enterobacteriaceae*
HAI	Hospital Acquired Infection
ICUs	Intensive care units
kb	kilo base
LIA	Lysine Iron Agar
MDR	Multi-Drug Resistant
MHA	Mueller-Hinton Agar
PCR	Polymerase chain reaction
SIM	Sulfide Indole Motility medium
TASH	Tikur Anbessa specialized Hospital
TSI	Triple Sugar Iron Agar

1 Introduction

Extended-spectrum β-lactamase-producing *Enterobacteriaceae* (ESBL-PE) has become a global challenge in infection control, resulting in prolonged hospital stays, increased hospital charges, and higher mortality and morbidity rates (Mahamat et al. 2019; Kumar 2017).

The β-lactamase production remains the most important mechanism of resistance to the β-lactam group of antibiotics and one group of these enzymes is called ESBLs (Chroma and Kolar 2010; Bukhari et al. 2016; Kumar 2017). ESBLs hydrolyze a wide range of cephalosporins including the oxyimino group of cephalosporins such as ceftriaxone, ceftazidime, cefotaxime, and the monobactam drugs such as aztreonam. They are found not to hydrolyze cephamycins and carbapenems, and their activity is inhibited by clavulanic acid (Kumar 2017; Laudy et al. 2017).

ESBLs are encoded by different genes inserted in genetic mobile elements, such as plasmids, that facilitate their spread between bacterial species (Chroma and Kolar 2010). The horizontal transmission of resistance genes through genetic mobile elements has an important role in the adaption of bacteria for various conditions in the hospital environments (Dziri et al. 2016a; Muzslay et al. 2017; Mahamat et al. 2019). ESBLs arise mostly due to mutations in β-lactamases encoded by the bla_{SHV}, bla_{TEM}, and bla_{CTX-M} genes (Mahamat et al. 2019; Ahmed et al. 2013).

ESBL producing bacteria have been detected from inanimate surfaces of hospitals that could contribute to their dissemination (Guet-Revillet et al. 2012; Engda et al. 2018). The detection of bacterial pathogens including ESBL producing bacteria from inanimate hospital environments is due to an innate ability of bacteria to survive on surfaces for long periods (Chemaly et al. 2014). For instance, *Klebsiella* spp. and *Escherichia*

coli, which are the predominant ESBL-PE, have been reported to survive up to 30 and 16 months, respectively, on inanimate dry surfaces (Guet-Revillet et al. 2012).

In Ethiopia, the rate of ESBLs on clinical isolates has been increased from time to time (Teklu et al. 2019; Legese et al. 2017; Desta et al. 2016). The increased rate of ESBL-producing bacteria in Ethiopia may be due to cross-transmission of multidrug-resistant (MDR) strains from patient to patient or from inanimate hospital environment to patients and from health care workers to patients and vice versa (Engda et al. 2018). The contaminated hospital environment is widely considered to be the principal cause of hospital-acquired infections (HAIs) and transmission of ESBL-PE (Facciolà et al. 2019).

Despite the recent worldwide spread of ESBL in *K. pneumoniae* and *E. coli* isolates from HAIs, their dissemination has been little studied especially in inanimate hospital environments in Ethiopia. This study was aimed at determining the magnitude of environmental contamination of ESBL-PE, antimicrobial susceptibility patterns and molecular characteristics of ESBL-PE, so as to a valuable input to control challenges in this direction.

2 Methods

2.1 Investigated Sites and Environmental Sampling

This descriptive cross-sectional study included 97 inanimate environmental swabs at Tikur Anbessa Specialized Hospital from June to July 2018. The samples were collected from five sites including ICUs of the Surgical, Neonatal, Pediatric, Medical, and Medical-Surgical wards of the hospital. The ICUs are hot zone for the emergency of resistant strains due to the less ventilation, the invasive procedures and the high antibiotic usage (Tajeddin et al. 2016; Bukhari et al. 2016). All samples were collected every morning after the cleaning was completed. Sampling sites around a bed in each ICU were chosen based on the frequency with which the surfaces were touched in each service. Samples were collected based on swab method (Rawlinson et al. 2019). The samples were collected on a single occasion, using pre-moistened sterile swabs from environmental surfaces and medical equipment including beds, monitors, linens, ventilators, suction machines, Lobby (furniture), work station, and sinks by suggestions from previous studies (Rocha et al. 2018; Chen et al. 2017). The samples were transported to laboratory within 2 h of collection. Analysis of swab samples was conducted at the post graduate Bacteriology Laboratory of the Department of Microbiology, Immunology and Parasitology, Addis Ababa, University.

2.2 Bacterial Cultures and Identification of Isolates

The swab samples were enriched overnight at 37 °C in brain heart infusion broth (BHI) (Merck, Germany) (Shamsizadeh et al. 2017). A loop full of bacterial growth was transferred to ESBL ChromoSelect Agar (SIGMA-ALDRICH) and MacConkey agar (Oxoid, UK) then incubated for 24 h at 37 °C to assess the ESBL production. Bacterial identification was achieved using specific colony color on ESBL ChromoSelect agar, MacConkey agar, and Gram staining combined with a panel of biochemical tests including Triple Sugar Iron agar (TSI), urea, citrate, Sulfide Indole Motility (SIM) medium, Lysine Iron Agar (LIA), mannitol, malonate and oxidase test (Supplementary Table S1) (Garcia 2010).

2.3 Antimicrobial Susceptibility Testing of ESBL-PE

Antimicrobial susceptibilities of ESBL producing *Enterobacteriaceae* isolates were determined using the disc diffusion method on Mueller-Hinton agar (MHA) (Oxoid, UK) based on Clinical and Laboratory Standards Institute (CLSI) performance standard methods and interpretive criteria (CLSI 2018). Isolates were tested for susceptibility to the following 13 antibiotics (in μg/

disk): ampicillin (10), amoxicillin/clavulanic acid (20/10), ceftriaxone (30), ceftazidime (30), cefotaxime (30), cefepime (30), aztreonam (30), meropenem (10), gentamicin (10), amikacin (30), ciprofloxacin (5), trimethoprim/sulfamethoxazole (1.25/23.75) and chloramphenicol (30). The different isolates tested were classified into sensitive (S), Intermediate (I) and Resistant (R) categories based on CLSI guidelines (CLSI 2018).

2.4 Phenotypic Confirmation of ESBL-PE

All the identified isolates were phenotypically confirmed for ESBL production using the Combination Disk (CD) method on Mueller-Hinton Agar (MHA) (Oxoid, UK). Briefly, a cefotaxime disc (30 μg) was placed 20 mm away from a disc containing cefotaxime-clavulanic acid (30/10 μg). When the inhibition zone between at least one of the combination discs and its corresponding single antibiotic disc differed by ≥5 mm, the strain was identified as an ESBL producer according to CLSI guidelines (Supplementary Tables S2 and S3) (CLSI 2018). MDR was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos et al. 2012).

2.5 Molecular Characterization of ESBLs Encoding Genes

2.5.1 DNA Extraction

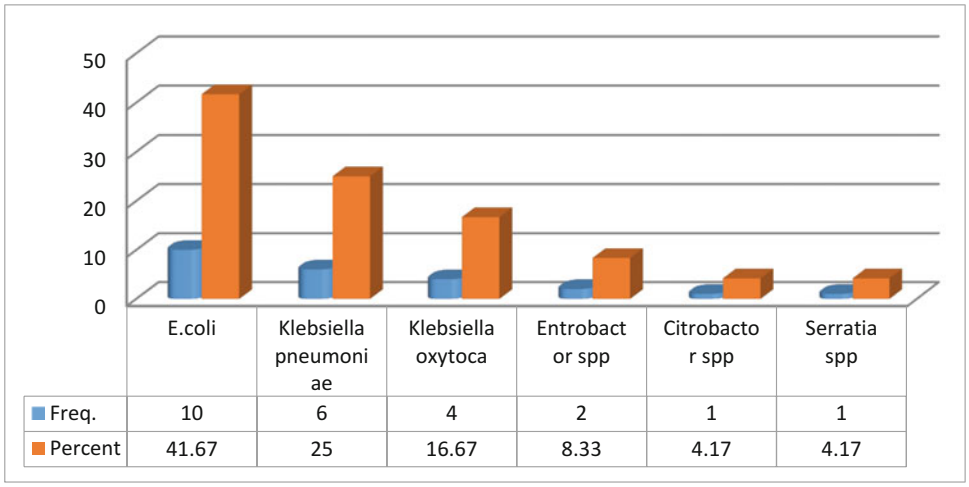
The DNA was extracted by the boiling lysis method as previously described by El-Badawy et al. (El-Badawy et al. 2017). Briefly, DNA was extracted from 3 to 5 fresh colonies of each isolate by incubation in a final volume of 300 μl of DNase-free water at 95 °C for 10 min followed by centrifugation at 14000 rpm for 5 min. The supernatant, which contains total genomic DNA, was used for further analysis. The quality and quantity of the extracted DNA were measured using Nanodrop (Thermo Scientific).

2.5.2 PCR Conditions and Interpretation

The presence of the bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ genes was assessed using a multiplex PCR method following the protocol (Mohammed et al. 2016) using specific Primers (Sigma-Aldrich, Germany) listed in Table 1. Briefly, the PCR was performed with approximately 300 ng template DNA, 0.2 μM of each primer, and 7.5 μl of 2 x QIAGEN Multiplex PCR Master Mix (QIAGEN) in a final volume of 15 μl. The cycling conditions were: 95 °C for 15 min, followed by 30 cycles of denaturation at 95 °C for 30s, annealing at 58 °C for 90s, elongation at 72 °C for 90s, and a final elongation step at 72 °C for 10 min by using T3000 thermocycler (Biometra). DNA samples from reference bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ positive strains were used as positive controls. The nuclease- free water was used as negative control in each amplification reaction. PCR products were visualized after electrophoresis at 120 V for 45 min on 1.5% agarose gels containing ethidium bromide. A 1 kb + DNA ladder (Thermo-fisher scientific, California) was used as size marker. The molecular analysis of ESBLs encoding genes was conducted at the Armauer Hansen Research Institute, Addis Ababa, Ethiopia.

Table 1 Primers used for detection of ESBLs genes by conventional PCR

Gene name	Primer name	Primer sequence (5′-3′)	Amplicon size (bp)	References
$bla_{CTX\text{-}M}$	**CTX-F**	CGCTGTTGTTAGGAAGTGTG	754	Darweesh, (2017)
	CTX-R	GGCTGGGTGAAGTAAGTGAC		
bla_{TEM}	**TEM-F**	TTTCGTGTCGCCCTTATTCC	403	Mohammed et al. (2016)
	TEM-R	ATCGTTGTCAGAAGTAAGTTGG		
bla_{SHV}	**SHV-F**	CGCCTGTGTATTATCTCCCT	293	Mohammed et al. (2016)
	SHV-F	CGAGTAGTCCACCAGATCCT		



	E.coli	Klebsiella pneumoniae	Klebsiella oxytoca	Enterobactor spp	Citrobactor spp	Serratia spp
Freq.	10	6	4	2	1	1
Percent	41.67	25	16.67	8.33	4.17	4.17

Fig. 1 Frequency of ESBL producing bacteria from inanimate environments at TASH, 2018

2.6 Quality Assurance

To ensure the quality of the result from different assays, internal quality assurance systems were in place for all laboratory procedures and double-checking of the result was done. Standard operating procedures (SOPs) were used for the specific purpose of all laboratory procedures. Quality control strains of *Escherichia coli* ATCC® 25922 and *Klebsiella pneumonia* ATCC 700603 were used to confirm the result of antibiotics, media, primers and to assess the quality of the general laboratory procedure (CLSI 2018).

2.7 Statistical Analysis

Data analysis was performed using SPSS version 25 software program (IBM Corporation, Armonk, NY, USA), and descriptive statistics (percentages or frequency) were calculated.

3 Results

ESBL producing *Enterobacteriaceae* were isolated in 24 of the 97 (24.7%) environmental samples, *Escherichia coli* (10/24, 41.7%), *Klebsiella pneumoniae* (6/24, 25%), and *Klebsiella oxytoca* (4/24, 16.7%) being the most predominant ESBL producing environmental isolates (Fig. 1).

3.1 Distribution of ESBL-PE Over Different Surfaces

The highest number of ESBL producing bacteria were obtained from bed linens 8(33.3%) followed by ventilators 5 (20.8%). Bed linens and ventilators were heavily contaminated by *Klebsiella pneumoniae* (50% vs 33.3%) and *E. coli* (30% vs 20%) (Table 2).

3.2 Distribution of ESBL-PE across Different ICU Surfaces

The Neonatal and Pediatrics ICU exhibited the highest ESBL producing *Enterobacteriaceae,* each with equal (29.2%, 7/24) (Table 2).

3.3 Antimicrobial Susceptibility Pattern of ESBL-PE

The Extended-spectrum β lactamase (ESBL) producing *Enterobacteriaceae* isolates obtained from this study were found to show significantly high resistance levels to ampicillin (100%),

Table 2 Distribution of ESBL-PE across different ICU surfaces

Variables	Distribution ESBL producing *Enterobacteriaceae* isolate n (%)				
	E. coli	*Klebsiella pneumoniae*	*Klebsiella oxytoca*	Others[a]	Total
Sampling points					
Bed	3	0	0	0	3(12.5)
Monitor	1	0	0	1	2(8.3)
Sink	0	0	0	2	2(8.3)
Suction machine	0	0	1	0	1(4.2)
Bed linens	3	3	2	0	8(33.3)
Lobby/furniture	1	1	0	1	3(12.5)
Ventilator	2	2	1	0	5(20.8)
Intensive care units					
Medical-surgical	3	0	1	2	6(25)
Neonatal	2	2	1	2	7(29.2)
Surgical	1	1	0	0	2(8.3)
Medical	1	1	0	0	2(8.3)
Pediatrics	3	2	2	0	7(29.2)
Total (N = 24)	10(41.7)	6(25)	4(16.7)	4(16.7)	24(100)

Notes: Others[a]: *Citrobacter* spp.; *Enterobacter* spp.; *Serratia* spp

ceftriaxone (91.7%), ceftazidime and cefotaxime each (83.3%). Similarly, a significant resistance level (79.2%) was also recorded for aztreonam, cefepime, and amoxicillin with clavulanic acid. A low resistance level was recorded for amikacin (25%) followed by meropenem (33.3%). Among the most common isolated ESBL, *E. coli* showed a higher rate of resistance to ampicillin (100%), ceftazidime, ceftriaxone, and cefotaxime each (90%). Furthermore, non-beta lactam antimicrobials such as ciprofloxacin and sulfamethoxazole-trimethoprim resistance were observed in 40% and 80% of *E. coli* isolates, respectively. *K. pneumoniae* revealed high resistance (100%) to ampicillin and ceftriaxone (Table 3).

3.4 Distribution of MDR Phenotypes

Of the total 24 ESBL producing *Enterobacteriaceae* isolates, 23(95.8%) were resistant to at least 3 antibiotics and 17 multi-drug resistance phenotypes were detected. The predominant MDR phenotype was observed in *E. coli* (10/10, 100%), *Klebsiella pneumonia* (6/6, 100%), *Enterobacter* spp. (2/2, 100%), and *Klebsiella oxytoca* (4/4, 100%). The most common MDR combinations found among *E. coli* isolates were against eight classes of antibiotics (Table 4).

3.5 Characterization of ESBL-Encoding Genes

Multiplex PCR detection of bla_{SHV}, bla_{TEM} and $bla_{CTX\text{-}M}$ genes in ESBL-PE showed that there were multiple occurrences of these genes in most of the isolates as shown in Fig. 2. The co-existence of SHV, TEM and CTX-M was seen in 16(80%) of the isolates while CTX-M and SHV in two of the isolates and CTX-M and TEM co-existed in two of the isolates. On the other hand, in four of the isolates none of the genes were detected. The CTX-M was the predominant genotype 20(35.7%) followed by SHV and TEM each with 18(32.1%) either alone or in combination (Table 5).

4 Discussion

The spread of ESBL-PE has been extremely rapid worldwide, indicating that continuous monitoring systems and effective infection control actions are unequivocally required (Ahmed et al. 2013; Guet-

Table 3 Antimicrobial susceptibility pattern of ESBL-PE from inanimate environments at TASH, 2018

Isolates	Antimicrobial agent's n (%)												
	Ptn	AMP	AZM	CTX	CRO	CTZ	FEP	AMC	CHL	MRP	AK	GEN	CIP	SXT
K. pneumoniae (n=6)	R	6(100)	4(66.7)	5(83.3)	6(100)	5(83.3)	5(83.3)	5(83.3)	3(50)	2(33.3)	3(50)	3(50)	2(33.3)	5(83.3)
	S	0(0)	2(33.3)	1(16.7)	0(0)	1(16.7)	1(16.7)	1(16.7)	3(50)	4(66.7)	3(50)	3(50)	4(66.7)	1(16.7)
E. coli (n=10)	R	10(100)	8(80)	9(90)	9(90)	9(90)	8(80)	7(70)	3(30)	3(30)	2(20)	5(50)	4(40)	8(80)
	S	0(0)	2(20)	1(10)	1(10)	1(10)	2(20)	3(30)	7(70)	7(70)	8(80)	5(50)	6(60)	2(20)
Enterobacter spp (n=2)	R	2(100)	2(100)	2(100)	2(100)	2(100)	2(100)	2(100)	2(100)	1(50)	0(0)	1(50)	2(100)	2(100)
	S	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(50)	2(100)	1(50)	0(0)	0(0)
K. oxytoca (n=4)	R	4(100)	4(100)	4(100)	4(100)	4(100)	4(100)	4(100)	3(75)	2(50)	1(25)	2(50)	3(75)	3(75)
	S	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(25)	2(50)	3(75)	2(50)	1(25)	1(25)
Citrobacter spp (n=1)	R	1(100)	1(100)	0(0)	1(100)	0(0)	0(0)	1(100)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	S	0(0)	0(0)	1(100)	0(0)	1(100)	1(100)	0(0)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)
Serratia spp (n=1)	R	1(100)	1(100)	0(00	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	S	0(0)	0(0)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)
Total (N=24)	R	24(100)	19(79.2)	20(83.3)	22(91.7)	20(83.3)	19(79.2)	19(79.2)	11(50)	8(33.3)	6(25)	11(45.8)	11(45.8)	18(75)
	S	0(0)	5(20.8)	4(16.7)	2(8.3)	4(16.7)	5(20.8)	5(29.8)	13(50)	16(66.7)	18(75)	13(54.2)	13(54.2)	6(25)

Abbreviations: *n* number of tested strains, % percentage, *R* Resistance, *S* Sensitive, *Ptn* Pattern, *AMP* Ampicillin, *AZT* Aztreonam, *CTX* Cefotaxime, *CRO* Ceftriaxone, *CTZ* Ceftazidime, *FEP* Cefepime, *AMC* Amoxicillin and clavulanic acid, *CHL* Chloramphenicol, *MRP* Meropenem, *AK* Amikacin, *GEN* Gentamicin, *CIP* ciprofloxacin, *SXT* Sulfamethoxazole + trimethoprim

Table 4 Multidrug-resistance profiles of ESBL-PE at TASH, 2018

MDR phenotype	Frequency, n (%)
E. coli	
AMP,CTX, AMC, MRP, AK, CIP, CHL, SXT	2(20)
AMP, CTX, SXT	2(20)
AMP, CTX, AMC	2(20)
AMP,CTX, AMC, AK, CIP, CHL, SXT	1(10)
AMP,CTX, AMC, MRP,AK,SXT	1(10)
AMP,CTX, AMC, SXT	1(10)
AMP,CTX, AMC, AK, CIP, SXT	1(100)
Total	10/10(100)
K. pneumonia	
AMP, CTX, AMC, MRP, AK, CIP, CHL, SXT	2(33.3)
AMP, CTX, AMC, AK, CHL, SXT	1(16.7)
AMP, CTX, AMC, GEN, SXT	1(16.7)
AMP,CTX, AMC, SXT	1(16.7)
Total	6/6(100)
K. oxytoca	
AMP,CTX, AMC, MRP, AK, CIP, CHL, STX	2(50)
AMP,CTX, AMC, AK, CIP, CHL, SXT	1(25)
AMP, CTX, AMC	1(25)
Total	4/4(100)
Enterobacter spp	
AMP, CTX, AMC, MRP, AK, CIP,CHL, SXT	1(50)
AMP, CTX, AMC, GEN, CIP, SXT	1(50)
Total	2(100)
***Citrobacter* spp**	1(100)
AMP, CTX, AMC	1(100)

Abbreviations: *AMP* Ampicillin, *CTX* Cefotaxime, *AMC* Amoxicillin and clavulanic acid, *CHL* Chloramphenicol, *MRP* Meropenem, *AK* Amikacin, *CIP* Ciprofloxacin, *SXT* Sulfamethoxazole + trimethoprim, *MDR* Multi Drug-Resistant

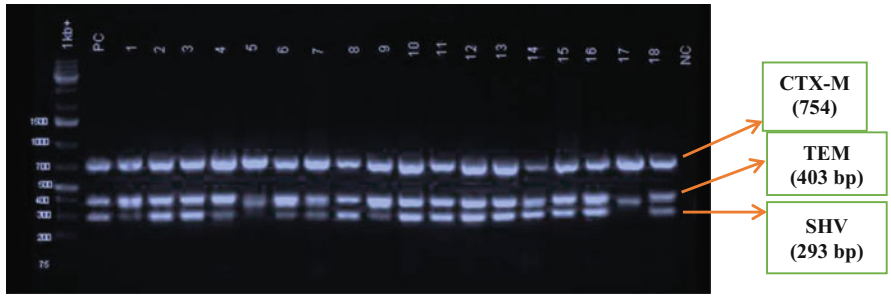


Fig. 2 Gel documentation image of the amplicons length showing samples from 1 to 18
Notes: *PC*, Positive control; 1kb+, DNA ladder; 1-18, positive samples; NC, Negative control; bp, base pair

Revillet et al. 2012). In our study, ESBL producing *Enterobacteriaceae* were isolated in 24 of the 97 (24.7%) environmental samples. In published reports, the percentage of inanimate hospital environments contaminated with ESBL are significantly varied ranging from 3% to 33% of surfaces in patients' rooms on regular hospitals wards, as reported from Gonder in Ethiopia (14.8%) (Engda et al. 2018), Tunisia (4%) (Dziri et al. 2016a), Israel (9%) (D'Agata et al. 1999),

Table 5 Distribution of SHV, TEM and CTX-M genes among ESBL-PE from inanimate environments at TASH, 2018

Isolates (n)	β-lactamase genes (n)
E. coli (9)	TEM/SHV/ CTX-M (7)
	CTX-M/SHV(1)
	CTX-M/ TEM (1)
Klebsiella pneumonia (5)	TEM/SHV/ CTX-M (4)
	CTX-M/ TEM (1)
Klebsiella oxytoca (3)	TEM/SHV/ CTX-M (2)
	CTX-M/SHV (1)
Enterobacter spp (2)	TEM/SHV/ CTX-M (2)
Citrobacter spp (1)	TEM/SHV/ CTX-M (1)

Tunisia (4%) (Guet-Revillet et al. 2012), UK (3.1%) (Muzslay et al. 2017) and Pakistan (33%) (Bukhari et al. 2016). These discrepancies could be due to differences in patient colonization load, hospital cleaning/disinfection protocols, study design including the timing of sample collection, and laboratory method used (Guet-Revillet et al. 2012; Dziri et al. 2016a; D'Agata et al. 1999; Freeman et al. 2014).

The high proportion of ESBL producers among *E. coli* and *K. pneumoniae* isolates in our study has agreed with studies conducted in Gondar, Ethiopia (Engda et al. 2018), Zimbabwe (Mbanga et al. 2018), Gaza in Palestine (Al Laham 2012), and Algeria (Manel et al. 2014). Given the fact that *Klebsiella* spp. and *Escherichia coli* can survive up to 30 and 16 months, respectively, on inanimate dry surfaces (Guet-Revillet et al. 2012). Moreover, isolation of such *Enterobacteriaceae* is highly indicative of fecal contamination and poor hand washing practices amongst health workers and patients (Sserwadda et al. 2018). Higher levels of ESBL-PE observed in our study could be attributed primarily to the use of ineffective disinfectants during surface cleaning, and inadequate uses of standard precautions such as hand hygiene and contact precautions, as well as migration of the organisms through air flow or other means. And thus it will be good to evaluate the performance of the disinfectants used in the hospital.

The most prevalent ESBL producer detected in the current study was *E. coli* (41.7%) as compared to *K. pneumoniae* (25%) similar reports from Gaza, Palestine (*E. coli* 40% vs *K. pneumoniae* 20%) (Al Laham 2012). In contrast to our result, different findings from most reports in other parts of the world ESBL production was more predominantly found among *Klebsiella* spp. isolates Zimbabwe (*Klebsiella* spp. 60% vs *E. coli* 40%) (Mbanga et al. 2018), Algeria (*K. pneumoniae* 48.5% vs *E. coli* 22.8%) (Manel et al. 2014), Gondar, Ethiopia (*K. pneumoniae* 42.1% vs *E. coli* 35.1%) (Engda et al. 2018), Sudan (*K. pneumoniae* 37% vs *E. coli* (0%) (Nurain et al. 2015) and on clinical isolates from another study in Ethiopia (*K. pneumoniae* 72.7% vs *E. coli* 22.7%) (Legese et al. 2017).

The Neonatal and Pediatrics ICU exhibited the highest ESBL producing *Enterobacteriaceae,* each with (29.2%, 7/24). This may lead to cross-infection between patients in the same ICU because patients present in these ICUs are likely to have reduced immune system due to age, illness, surgical and mechanical manipulation, and/or the use of immune-suppressors and other therapeutic drugs, all of which increasing patients susceptibility to infections (Abreu et al. 2013; Yusuf et al. 2017).

The linens, ventilators, beds, and sink were inanimate surfaces observed to be associated with a variable degree of ESBL producing *Enterobacteriaceae* positivity, with 33.3%, 20.8%, 12.5%, and 8.3%, respectively. The identification of ESBL producing bacteria on linens, sink, bed, and other environmental surfaces is consistent with reports from the literature (Guet-Revillet et al. 2012; Engda et al. 2018; Muzslay et al. 2017; D'Agata et al. 1999). It is generally assumed that ESBL producing Gram-negative bacteria require moist or damp sites for enhanced

longevity (Muzslay et al. 2017; Manel et al. 2014). Moreover, bed linens are considered to be high patient-contact surfaces and therefore the detected pathogens might have been shed by the infected/colonized patients occupying the particular beds.

In regards to the level of antimicrobial resistance among our ESBL producing isolates, a significantly high resistance level was recorded to penicillin groups such as ampicillin (100%) and cephalosporin groups such as ceftriaxone (91.7%), ceftazidime (83.3%), cefepime (79.2%), azetronome (79.2%) and cefotaxime (83.3%). These results were mostly comparable with results from other studies conducted in Ethiopia (cefpirome, cefpodoxime, ceftazidime, ceftriaxone, and amoxicillin with clavulanic acid each with 100% resistance level) (Engda et al. 2018) and Algeria where ampicillin resistance level was reported at 98.1% (Manel et al. 2014). Probably lack of antibiotic resistance screening and confirmatory testing before using these drugs could be responsible for the accumulation of such a high level of resistance among ESBL producing bacteria in the hospital environments.

A high resistance rate was also recorded for ESBL-producing isolates for non-beta-lactam antimicrobials such as cotrimoxazole (75%), ciprofloxacin (45.8%), and chloramphenicol (50%). This was in close agreement with three other studies done in Ethiopia, ciprofloxacin (56%) (Engda et al. 2018), from clinical isolates in Addis Ababa, cotrimoxazole 77%, ciprofloxacin 46.3% (Teklu et al. 2019) and cotrimoxazole (83.6%), Chloramphenicol (61.8%) (Legese et al. 2017). The most active drugs for ESBL-producing isolates were amikacin (75%). The low resistance rate of amikacin in our study could be explained by the dearth in the usage of amikacin in our study setting.

Reports from other studies showed that multidrug resistance to the commonly prescribed antimicrobial agents are more common among ESBL producers found from inanimate hospital surface (Zenati et al. 2016; Dziri et al. 2016b). Hence, unwise use of antibiotics can inadvertently favor the emergence of MDR bacterial strains (Dziri et al. 2016a, Yusuf et al. 2017). The finding from the current study showed that (23/24, 95.8%) of the ESBL producing bacterial isolates were resistant to at least 3 antibiotics. This in turn indicates MDR *Enterobacteriaceae* is rampant at the hospital environments. This finding is higher than reports from Northwest Ethiopia (75%) (Getachew et al. 2018), Zimbabwe (75%) (Mbanga et al. 2018), and Iran (79.4%) (Tajeddin et al. 2016).

The predominant genotype of ESBL-PE has changed from TEM and/or SHV to CTX-M (Mohammed et al. 2016; Darweesh 2017). The result from this study also showed CTX-M was the predominant genotype 20(35.7%) followed by SHV and TEM each with 18(32.1%) either alone or in combination. Several studies from Africa and abroad have also reported that the CTX-M gene is now replacing TEM and SHV genes as the commonest ESBL type (El-Badawy et al. 2017; Mohammed et al. 2016; Darweesh 2017). The significance of the finding of organisms possessing multiple genes is more likely to have multidrug resistance and more likely to tend widespread nosocomial transmission (Mohammed et al. 2016; Mahamat et al. 2019; Darweesh 2017).

5 Conclusions

The findings from the currents study showed that the appearance of ESBL-PE among ICUs hospital environment poses a serious threat for the control of healthcare associated infections. Occurrence of MDR is found to be more common among ESBL producers, showing the need for policies to prevent misuse and overuse of antimicrobial agents. Our results may be indicative evidence that ESBL-PE is possibly contributing to HAIs and MDR strain dissemination in the hospital environment. The co-existence of SHV, TEM, and CTX-M from ESBL-PE isolates were major concern and highlights the need for further surveillance in this area. Hence, we recommended that large-scale investigations are needed to assess clonal relationship between the inanimate surface and clinical strains which may contribute for a better understanding on the correlations.

Moreover, we recommended application of Whole genome sequencing (WGS) of the MDR bacterial isolates to detect a larger number of antimicrobial resistance genes by using large sample size from different hospitals to give better figurative data.

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Dataset The datasets supporting the conclusions of this article are included within the article and its additional files.

Author Contributions All authors made a significant contribution to the work reported, SS, WE, TA, AS conceived and designed the experiments, AM, WM, TA contributed of reagents/materials, SS, AS, TW performed the experiments, SS, WE, AM, TA, TW, AS execution, acquisition of data, analysis and interpretation; SS, ZD, WM, TW, AM, TA, TW, AS took part in drafting, critically reviewing the article; All authors gave final approval of the version to be published.

Ethics The study protocol was approved by the Department of Microbiology, Immunology and Parasitology Research Ethics Review Committee (DRERC), College of Health Sciences, Addis Ababa University (Ref. no. DRERC/17/18/02-G). Written permission letter was obtained from the clinical service director of Tikur Anbessa Specialized Hospital prior to initiation of sample collection.

Conflict of Interest Statement The authors report no conflicts of interest in this work.

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Distribution and Antibiotics Resistance Pattern of Community-Acquired Methicillin-Resistance *Staphylococcus aureus* in Southwestern Nigeria

Ibukunoluwa Olayinka Oginni and Ademola Adetayo Olayinka

Abstract

Background Methicillin-resistant *Staphylococcus aureus* is a global public health challenge and there is a continuous increase in community-acquired infections among people in different geographical location. We sought the distribution and antibiotics pattern of community-acquired methicillin-resistant *Staphylococcus* isolates among apparently healthy residents of Ibadan, Southwestern Nigeria.

Methods Seven hundred (700) healthy volunteers residing in Ibadan metropolis, Nigeria, were enrolled in this study. Isolates from the nasal swabs were aseptically collected and characterized using standard and established microbiological methods, which included growth and fermentation on mannitol salt agar, colonial morphology, Gram-staining reaction, Microbact™ 12S identification kit and confirmed with 16SrRNA. After identification of the isolates, antimicrobial susceptibility test was performed on Mueller-Hinton agar by modified Kirby-Bauer disc diffusion method and the presence of *mecA* and *nuc* genes were detected via polymerase chain reaction assay.

Results Prevalence of *Staphylococcus aureus* nasal carriage and Methicillin-resistant *Staphylococcus* in this study was 31.9% and 9.43% respectively. The residents of Ibadan North local government area (Fisher's Exact = 1.8962, $P = .028$) and Egbeda local government area (Fisher's Exact = 2.7222, $P = .006$) are likely to carry Methicillin-resistant *Staphylococcus* than any other local government area in Ibadan, Nigeria. The antimicrobial resistance patterns of the isolates revealed high resistance to Oxacillin (96.9%). Most of the isolates were sensitive to vancomycin (92.4%). Polymerase chain reaction analysis showed that *mecA* gene was present in all 66 (100%) Methicillin-resistant *Staphylococcus aureus* isolates. Male-gender ($\varkappa^2 = 8.849$, $P = .003$), Adults; 40–50 years old ($\varkappa^2 = 9.842$, $P = .002$), low educational background ($\varkappa^2 = 36.817$, $P < .001$), recent hospital visitation ($\varkappa^2 = 8.693$, $P = .003$) are some of the factors that are observed in this study to be associated with Methicillin-resistant *Staphylococcus* infection.

Conclusion Our findings revealed the relatively high frequency of nasal carriers of

Supplementary Information: The online version of this chapter (https://doi.org/10.1007/5584_2021_658) contains supplementary material, which is available to authorized users.

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Methicillin-resistant *Staphylococcus aureus* among the apparently healthy residents of the studied area and the advent of multidrug resistance among these isolates. Our study also supports previous findings on male-gender and low educational background as risk factors of *S. aureus* carriage. The need for rational chemotherapy, routine detection and regular surveillance of Methicillin-resistant *Staphylococcus* to limit its spread and reduce treatment failures is important.

Keywords

Antibiotic resistance · mecA gene · MRSA · Multidrug resistance · Nasal carriers · Nigeria

1 Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as an important pathogen in human medicine. MRSA was first reported in 1961, soon after the introduction of methicillin into human medicine to treat penicillin-resistant staphylococci in nosocomial infections (Conly and Johnston 2003; Lakhundi and Zhang 2018). Methicillin resistance is of great importance because it is conferred by the presence of the *mecA* gene that resides on a staphylococcal chromosomal cassette (SCC), which encodes for the production of an altered penicillin-binding protein (PBP; PBP2a or PBP2) that has a reduced affinity for all beta-lactam antimicrobials. In the past five decades, the incidences of both nosocomial and community-acquired *S. aureus* (CA-MRSA) infections have increased, while antibiotics treatment options are increasingly hampered by the spread of MRSA (Davis et al. 2007; Yarovoy et al. 2019). MRSA are resistant to a broad range of antimicrobials and are also frequently resistant to most of the commonly used antimicrobial agents such as aminoglycosides, macrolides, chloramphenicol, tetracycline, and fluoroquinolones which make MRSA particularly difficult to treat.

From the systematic review carried out by Abubakar and Sulaiman (2018), the prevalence of MRSA infection in Nigeria was about 50%. Poor infection control, inappropriate use of antibiotics and poor implementation of the developed National action plan for antimicrobial resistance could explain the rising trends of MRSA in Nigeria (FMoH 2017). However, efforts are currently being made to implement such interventions in Nigeria. The emergence of CA-MRSA has changed the epidemiology of *S. aureus* as reported by several studies (Iwao et al. 2017; Oliveira et al. 2018; Wang et al. 2016). Many studies have characterized *S. aureus* and MRSA isolates from individuals at some selected communities and hospitals but there is a paucity of information on the distribution of staphylococcal nasal carriage and MRSA among a group of communities in southwestern Nigeria particularly, in Ibadan. Therefore, we sought to determine the distribution, risk factors, and the antibiotic resistance patterns of MRSA to commonly prescribed antibiotics in these localities. This would be useful in choosing empirical therapy for the treatment of CA-MRSA infections and the enactment of infection control guidelines.

2 Methods

2.1 Ethical Approval

Ethical approval was sought from the Health Research Ethics Committee (HREC) of the Institute of Public Health, Obafemi Awolowo University, Ile-Ife, Nigeria. We obtained informed consent from all participants or their legal guardians and confidentiality of all participants and premise data was strictly maintained.

2.2 Study Population and Collection of Samples

This was a cross-sectional, multicenter study using a proportionate stratified random sampling

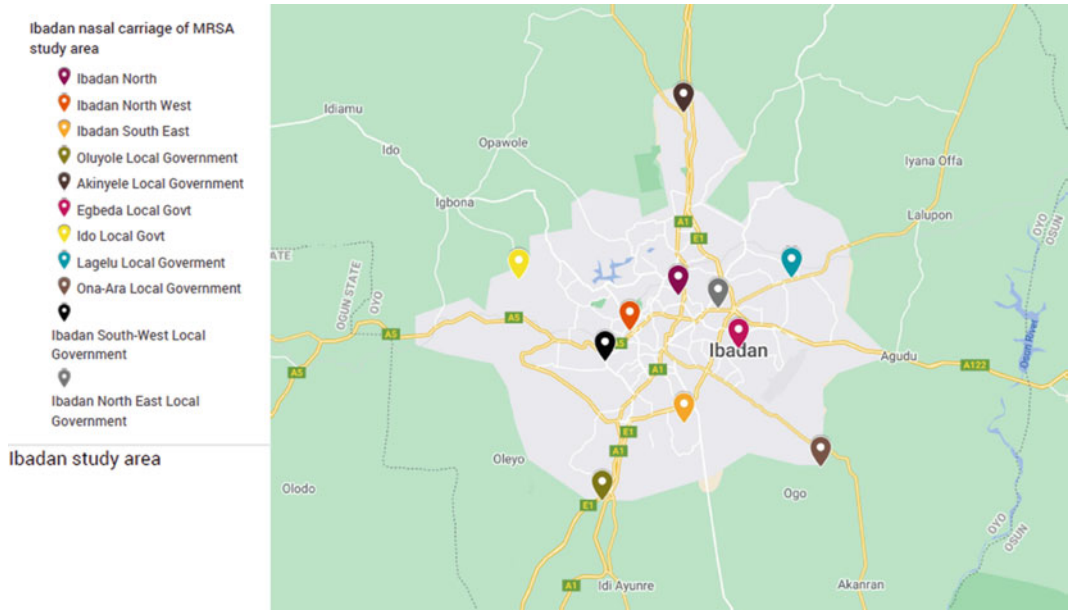

Fig. 1 Map indicating the location of the study area (Olayinka 2021)

technique. Ibadan city is the state capital of Oyo-State with over 3.7 million inhabitants. The participants were recruited from the 11 local government areas (LGAs) in Ibadan, Nigeria for a period of 9 months (Fig. 1). Nasal specimens were collected by streaking both the anterior nares of each participant using sterile swabs (Copan Diagnostics, Corona, CA, USA) moisture with sterile normal saline. The samples were transported to the laboratory aseptically within 2 h of collection.

2.3 Isolation and Identification of *Staphylococcus aureus*

All nasal swabs were inoculated onto Mannitol Salt Agar and Blood Agar (Oxoid Ltd., Hampshire, England) aseptically and incubated aerobically at 37 °C for 24–48 h. Discrete colonies of *Staphylococcus aureus* were sub-cultured on Nutrient agar (Oxoid Ltd., Basingstoke, Hampshire, England) plates incubated aerobically at 37 °C for 24 h to obtain pure culture and for further analyses. Each isolate was identified using Gram-staining, Cowan & Steel method of bacteria identification, Microbact™ 12S identification kit and confirmed via 16SrRNA gene detection.

2.4 Antimicrobial Sensitivity Testing

The susceptibility of recovered *S. aureus* isolates to various antibiotics were determined according to Kirby-Bauer disc diffusion technique and the (Clinical and Laboratory Standards Institute 2021) guideline was used to interpret the result. The tested antibiotics include Tetracycline (30 μg), Gentamicin (10 μg), Clindamycin (2 μg), Erythromycin (15 μg), Oxacillin (1 μg), Ceftaroline (30 μg), Co-trimoxazole (25 μg), Linezolid (30 μg), Vancomycin (30 μg), Cefoxitin (30 μg), and Ceftriaxone (30 μg) (Oxoid Ltd., Basingstoke, United Kingdom). We used cefoxitin discs (30 μg) (Oxoid Ltd., Basingstoke, United Kingdom) to phenotypically screen for methicillin-resistance). *S. aureus* ATCC 25923 was used for quality control.

2.5 Genomic DNA Isolation and Detection of *mecA* and *nuc* Genes

Promega (Madison, USA) genomic DNA extraction kit was according to the manufacturers' instructions using aseptic precautions. Polymerase Chain Reaction (PCR) assay was carried out for *mecA* (for detection of methicillin resistance) and *nuc gene* (for detection of *S. aureus*). The primer sequenced were as follows *mec-A1* (5′-AAA ATC GAT GGT AAA GGT TGC C-3′), *mec-A2* (5′- AGT TCT GCA GTA CCG GAT TTG C- 3′) and *nuc-A1* (5′- GCG ATT GAT GGT GAT ACG GTT-3′), *nuc-A2* (5′- AGC CAA GCC TTG AAC GAA CTA AAGC- 3′ (David et al. 2010).

2.5.1 Questionnaire Design

We developed and administered a structured questionnaire. Three independent reviewers were selected to validate the questionnaire; to assess the content validity, clarity, ease of response, scope, and face validity of the questions, We also obtained the participants' demographic characteristics (gender, education, and age group) and information on risk factors (recent antibiotics use, handwashing frequency, educational background of participants, etc.) for predisposition to colonization (Supplementary file 1).

2.5.2 Statistical Analysis

The data was summarized using Microsoft excel 2016 and subjected to further statistical analysis using Chi-Square and Fisher's Exact Probability Test in Epi-Info V.7.0 (CDC, Atlanta, USA). Inferences were made based on computed Prevalence ratios, their 95% confidence intervals and p-values. The level of significance was set at $p < 0.05$.

3 Results

Seven hundred (700) participants were included in this study from their respective LGAs; 56% (392/700) males and 44% (308/700) female participants while the age range (30–40 years) had the highest frequency of participants 20.1% (141/700). Most of the participants (62.6%, n = 438/700) had no formal/primary education (Table 1). The prevalence of *S. aureus* nasal carriage and MRSA in this study was 31.9% (223/700) and 9.43% (66/700) respectively. MRSA was well distributed in all the eleven (11) LGAs in the study area with the highest prevalence in Egbeda LGA (28.6%, n = 18/63). Results showed that methicillin-resistant *S. aureus* is significantly associated with participants that reside in Ibadan North LGA (FE = 1.8962, $P = .028$) and Egbeda LGA (FE = 2.7222, $P = .006$) (Table 2).

Table 1 Description of the study participants (n = 700)

Variables	Frequency (%)
Gender	
Female	392 (56.00)
Male	308 (44.00)
Age	
1–10	63 (9.00)
>10–20	83 (11.86)
>20–30	127 (18.14)
>30–40	141 (20.14)
>40–50	140 (20.00)
>50–60	99 (14.14)
>60–70	47 (67.14)
Educational background	
Secondary & tertiary education	262 (37.43)
No formal education & primary education	438 (62.57)

Table 2 Profile of *Staphylococcus aureus* nasal carriage (SANC) and methicillin-resistance *Staphylococcus aureus* (MRSA) isolates from Ibadan

Local govt. area N = 700	Total participants N (%)	SANC, n (%)	MRSA, n (%)	FE (P-value)
Ibadan North	190 (27.14)	71 (37.36)	31 (16.32)	**1.8962 (0.0281)***
Ibadan North East	73 (10.43)	12 (16.44)	2 (2.74)	0.5495 (0.7437)
Ibadan North West	34 (4.90)	6 (17.65)	1 (2.94)	0.5564 (1.0000)
Ibadan South East	60 (8.57)	21 (35.00)	4 (6.67)	0.6206 (0.4659)
Ibadan South West	63 (9.00)	27 (42.86)	3 (4.76)	0.3457 (0.1058)
Oluyole	46 (6.50)	21 (45.65)	2 (4.35)	0.3006 (0.1205)
Akinyele	55 (7.79)	6 (10.91)	1 (1.82)	0.5564 (1.0000)
Egbeda	63 (9.00)	27 (42.85)	18 (28.57)	**2.7222 (0.0059)***
Ido	23 (3.24)	3 (13.04)	1 (4.35)	1.1282 (1.0000)
Lagelu	33 (4.71)	7 (21.21)	1 (3.03)	0.4747 (0.6872)
Ona-Ara	60 (8.57)	22 (36.67)	2 (3.33)	0.2855 (0.1238)

FE Fisher's exact, *SANC* Staphylococcus aureus nasal carriage, *MRSA* Methicillin-resistant Staphylococcus aureus
*p < 0.05 (i.e. statistically significant)

The susceptibility pattern of nasal *S. aureus* to different antibiotics is presented in Table 3. Most of the MRSA were resistant to other antibiotics such as Oxacillin (96.9%), clindamycin (62.1%), Co-trimoxazole (59.1%) as revealed in Fig. 2. This study showed that nasal *Staphylococcus aureus* are highly multidrug-resistant as 54% were resistant to two different classes of antibiotics while 24% were resistant to three or more different classes of antibiotics. All the 66 (100%) MRSA isolates harboured the *mecA* gene (Fig. 3). In addition, 18 (27.3%) of the recovered MRSA possess the *nuc* gene (Fig. 4).

The result of this study showed that participants between 40–50 years old (34.5%, $\varkappa^2 = 9.842$, $P = .002$) and the male participants (35.3%, $\varkappa^2 = 8.849$, $P = .003$) were more likely to be carriers of MRSA (Table 4). Some of the factors that are associated with MRSA infection were tested in this study; the participants that visited the hospital recently (39.7%, $\varkappa^2 = 8.693$, $P = .003$), used antibiotics recently (61.7%, $\varkappa^2 = 7.556$, $P = .006$) and those with low educational background (3.9%, $\varkappa^2 = 36.817$, $P < .001$) are more likely to be carriers of MRSA as presented in Table 4.

4 Discussion

The epidemiological knowledge of Methicillin-resistant *Staphylococcus aureus* (MRSA) infection is very important for appropriate decision-making in the treatment of infections. Since at least 1978 when we have the first reported case of MRSA in Africa (Scragg and Appelbaum 1978), we have been attending to an increase in the number of infection episodes in healthy individuals (both adults and children) in the community (Abubakar and Sulaiman 2018). According to the review done by Abdulgader et al. (2015), only fifteen (15) out of the fifty-four (54) African countries are with reports on the molecular epidemiology of MRSA. Additionally, despite the global challenge on the burden of disease caused by CA-MRSA, in Nigeria, only a few isolated studies describing MRSA with typical community backgrounds were reported (Ghebremedhin et al. 2009). In this study, we report for the first time the prevalence, risk factors and the epidemiology of CA-MRSA infections in Ibadan as well as determine the antibiotic resistance indices of MRSA in different communities in Ibadan, Nigeria.

It has been reported that all beta-lactam antibiotics have poor affinity when penicillin-binding protein (PBP) is altered; it will be difficult to kill such microorganisms when exposed to therapeutic concentration. Methicillin resistance is mediated among *Staphylococcus aureus* by the penicillin-binding protein encoded by the *mecA* gene (Ito et al. 2014). The overall prevalence of CA-MRSA in this study was 9.43%. The MRSA prevalence reported in this study is higher than what was reported (4%) by Ajani et al. (2020) among students of a private institution in Ogun-State, Nigeria.

Table 3 Resistance profile of Nasal *S. aureus* from healthy population in Ibadan

Antibiotics	Concentrations (μg)	Resistance n (%)	Intermediate n (%)	Susceptible n (%)
Tetracycline	30	29 (13.00)	3 (1.35)	191 (85.65)
Gentamicin	10	14 (6.28)	7 (3.14)	202 (90.58)
Clindamycin	2	126 (56.50)	43 (19.28)	54 (24.22)
Erythromycin	15	85 (38.12)	35 (15.69)	103 (46.19)
Oxacillin	1	187 (83.86)	18 (8.07)	18 (8.07)
Ceftaroline	30	32 (14.35)	6 (2.69)	185 (82.96)
Co-trimoxazole	25	180 (80.72)	16 (7.17)	27 (12.11)
Linezolid	30	47 (21.08)	11 (4.93)	165 (73.99)
Vancomycin	30	8 (3.59)	9 (4.04)	206 (92.38)
Cefoxitin	30	91 (40.81)	0 (0.00)	132 (59.19)
Ceftriaxone	30	35 (15.70)	24 (10.76)	164 (73.54)

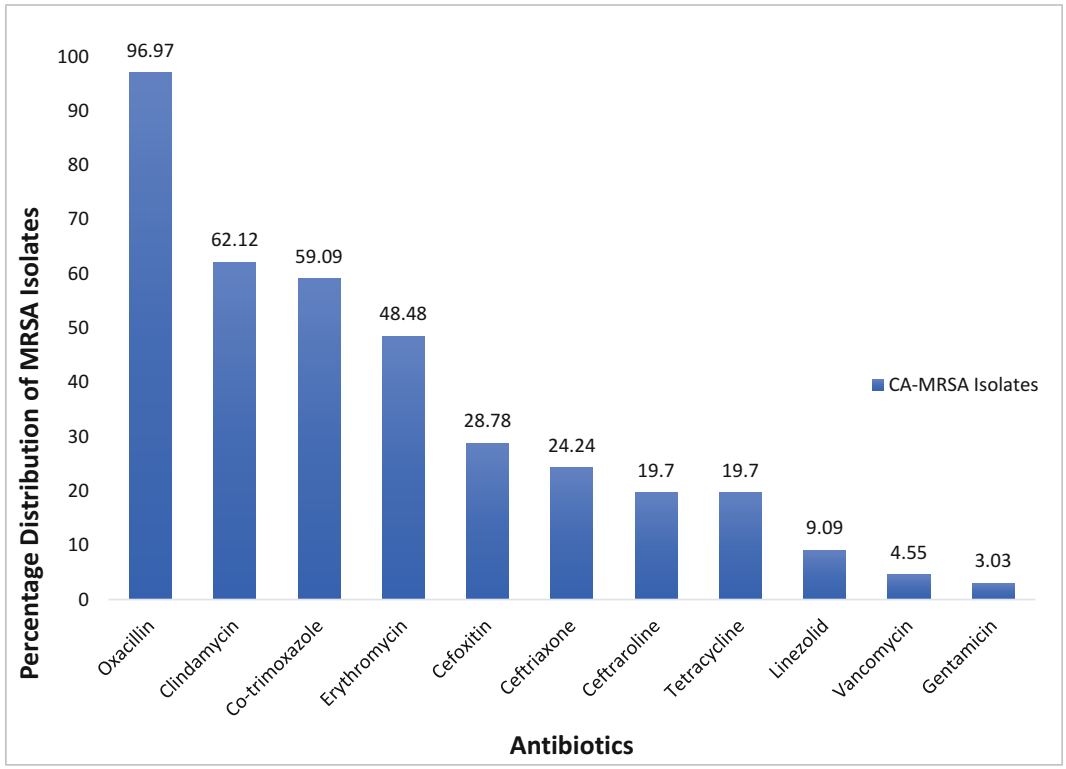

Fig. 2 Antimicrobial resistance pattern of CA-MRSA in Ibadan

We detected *nuc* genes of *S. aureus* in only 27.3% of the MRSA isolates this is probably due to the differences in the nucleotide sequence among the *nuc* genes caused by some mutation or the absence of *nuc* gene in some *S. aureus* strains (Sahebnasagh et al. 2014). The difference between the phenotypic and genotypic detection of *S. aureus* strains made it clear that, the method of identifying only *nuc* genes in *S. aureus* is not sufficient. Therefore, both phenotypic and genotypic methods should be conducted for the identification of *S. aureus* strain. Methicillin-resistant

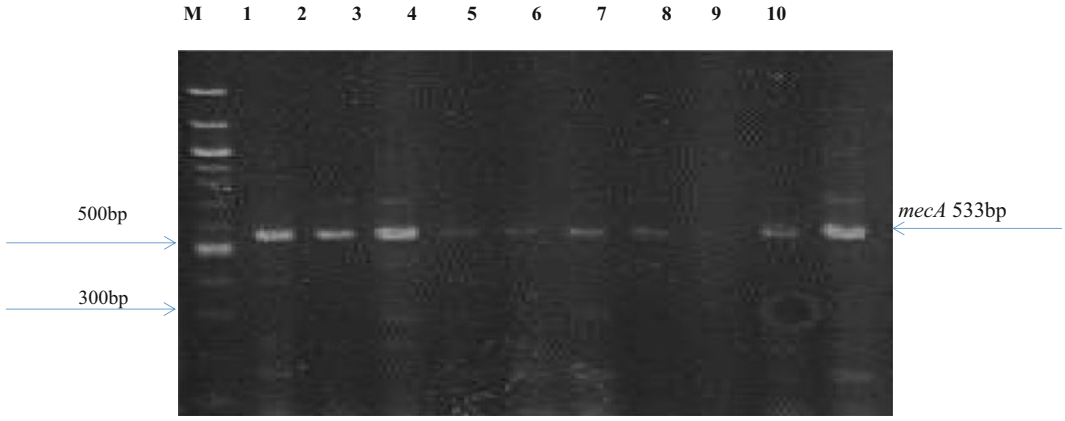

Fig. 3 PCR amplification of *MecA* gene (533 bp), 16-10-2020
Lane M: DNA molecular size marker (100 bp ladder), Lane 1: ATCC 33591 (positive control), Lane 2: Isolate 14; Lane 3: Isolate 28; Lane 4: Isolate 35; Lane 5: Isolate 38; Lane 6: Isolate 48; Lane 7: Isolate 55; Lane 8: Isolate 62; Lane 9: Isolate 69; Lane 10: Isolate 71.

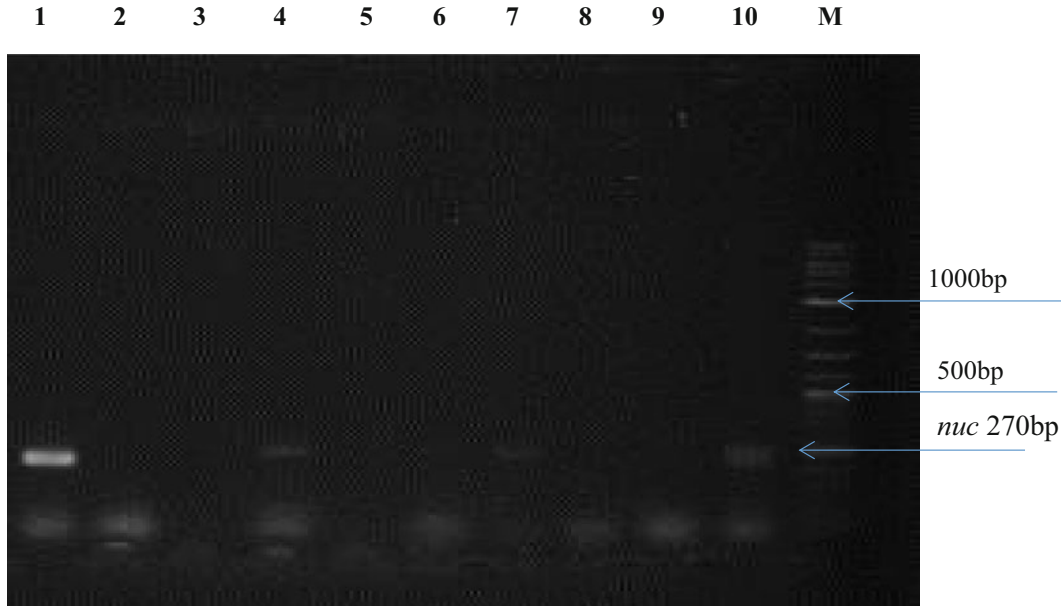

Fig. 4 Lane 1: Isolate 14, Lane 2: Isolate 16; Lane 3: Isolate 19; Lane 4: Isolate 22; Lane 5: Isolate 25; Lane 6: Isolate 27; Lane 7: Isolate 35; Lane 8: Isolate 36; Lane 9: Isolate 43; Lane 10: ATCC 29213 (positive control), Lane M: DNA molecular size marker (100 bp ladder)

Staphylococcus aureus isolates were obtained from all the 11 LGAs in Ibadan, Nigeria. Egbeda and Ibadan North LGAs presented the highest MRSA prevalence in this study. Individuals that reside in either of these LGAs (Ibadan North; FE = 1.8962, P = .028 or Egbeda; FE = 2.7222, P = .006) are more likely to carry MRSA than those in other areas. This is because the two LGAs are among the most populated LGAs in Ibadan with shared border and linked

Table 4 Bivariate analysis of risk factors for MRSA

Variable	Total participants N = 700, N (%)	SANC, n (%)	MRSA, n (%)	χ^2 (P-value)
Gender				**9.842 (0.002)***
Male	392 (56.00)	142 (36.22)	49 (34.50)	
Female	308 (44.00)	81 (26.21)	17 (20.99)	
Recent antibiotics used				**7.556 (0.006)***
Yes	205 (29.29)	47 (22.92)	29 (61.70)	
No	495 (70.71)	176 (35.56)	37 (21.02)	
Recent hospital visit (in the last 3 months)				**8.693 (0.003)***
Yes	220 (31.43)	78 (35.45)	31 (39.74)	
No	480 (68.57)	145 (30.21)	35 (24.14)	
Household members who care for sick people				0.137 (0.711)
Yes	15 (2.14)	3 (20.00)	1 (33.33)	
No	685 (97.86)	221 (32.26)	65 (29.41)	
Hand washing frequency				1.298 (0.255)
Often	576 (82.29)	197 (34.20)	51 (25.89)	
Seldom	124 (17.71)	26 (20.97)	15 (57.69)	
Educational background				**36.817 (<0.001)***
Secondary & tertiary education	262 (37.43)	51 (19.47)	2 (3.92)	
No formal education & Primary education	438 (62.57)	172 (39.27)	64 (37.21)	
Smoking habits[a]				
Ex-smoker	63 (9.00)	26 (41.27)	6 (23.08)	0.001 (0.978)
Current smoker	141 (20.14)	40 (28.37)	11 (27.50)	0.547 (0.459)
Non-smoker	496 (70.86)	157 (31.65)	49 (31.21)	0.404 (0.525)

χ^2 = Chi square, *S. aureus* nasal carriage (SANC)
*$p < 0.05$ (i.e. statistically significant)
[a]multivariate analysis was used

roadways (NPC 2006; Olayinka 2021). This result is similar to the previous MRSA prevalence range of 14.3–37% that has been reported in different communities in Nigeria (Akerele et al. 2015; Bale et al. 2019; Egwuatu et al. 2016; Ghebremedhin et al. 2009; Taiwo et al. 2005). This is not surprising as the MRSA prevalence varies greatly with geographical location and studied population (Bell et al. 2002).

Antimicrobial resistance (AMR) is one of the major threats posed by microorganisms in this twenty-first century. Despite the serious efforts employed to control AMR by aggressive infection control methods, MRSA has become one of the most frequent cause of hospital and community-acquired infections globally. MRSA has always been one of the major pathogens that possess the ability to develop resistance to newly developed antimicrobial agents (Joo et al. 2017). In this study, *Staphylococcus aureus* nasal carriage exhibited resistance to Oxacillin, Co-trimoxazole and Clindamycin but susceptible to Vancomycin, Gentamicin, Tetracycline, Ceftaroline and Linezolid in varying degrees. The MRSA isolates were also resistant to multiple antibiotics. The antimicrobial resistance patterns of the isolates revealed high (80%) multidrug resistance (resistance against at least 2 different classes of antimicrobials) rate among the MRSA isolates. Shariati et al. (2020) reported a global increase in the prevalence of vancomycin resistance in their systemic review of global prevalence and distribution of vancomycin-resistant *Staphylococcus aureus*. Therefore, the 7.6%prevalence of vancomycin resistance in this study is not surprising although it remains a major global

public health challenge. The relatively high resistance prevalence observed in both Ceftaroline and linezolid in this study are comparable with the rate of resistance reported in a neighboring town by Osinupebi et al. (2018). Many factors can be linked to the high resistance of this organism to antibiotics in these communities. Such factors include self-medication, availability and use of antibiotics without prescription, irrational consumption rate of antibiotics, over the counter accessibility without prescription and sales of fake or substandard drugs, unrestricted use of antimicrobials in farm animals (including poultries and fisheries), and transmission of resistant strains between individuals within the community (Akerele et al. 2015; Elimam et al. 2014).

One of the main reason for *mecA* gene screening by polymerase chain reaction (PCR) technique is to compare the results of antibiotic susceptibility by disc diffusion method with gene analysis results in *Staphylococcus aureus* isolates. Not all MRSA strains may be detectable with phenotypical methods as some *mecA* genes may be heterogeneously expressed. In this study, all the MRSA isolates that were cefoxitin resistant were also positive for this gene detection method confirming that they are all methicillin-resistant *Staphylococcus aureus.* The study showed a strong correlation between genotypic and phenotypic analysis and it is consistent with previous studies that reported a perfect association between the results obtained by the phenotypic antibiotic resistance determination and PCR- based assays (Bale et al. 2019; Ito et al. 2014; Strommenger et al. 2006).

The present study showed a high proportion of CA-MRSA in the male-gender than in female which agrees with previous studies (Abroo et al. 2017; Mehraj et al. 2014; Skramm et al. 2011). The microbial differences between male and female could be due to physiological factors or anatomical differences between genders (Giacomoni et al. 2009). Our findings that males were more likely to carry MRSA is consistent with other studies, which indicate a gender-specific risk factor (Andersen et al. 2013; Assafi et al. 2015; Gorwitz et al. 2008; Graham et al. 2017; Mehraj et al. 2014; Skramm et al. 2011). Some studies have reported the association of MRSA among adults and elderly as revealed in this study (Andersen et al. 2013; Gorwitz et al. 2008). This study also revealed that individuals with certain risk factors (age, male gender, recent use of antibiotics, recent hospital visitation, and level of education) were more likely to be carriers of MRSA (Abroo et al. 2017; Graham et al. 2017).

5 Conclusion

In conclusion, the strongest risk factors of CA-MRSA were male gender, low educational background, recent antibiotics used, and hospital visits. The relatively high prevalence of CA-MRSA in this study is a cause for concern. CA-MRSA showed a high resistance burden and individuals who are harboring these isolates can act as reservoirs; this may negatively influence the treatment of CA-MRSA infections. The continuous surveillance of CA-MRSA is essential to prevent transmission of *Staphylococcus aureus* from the infected carriers to others also to apply effective therapeutic options for their treatment.

Competing Interests Authors have declared that no competing interests exist.

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Ultrastructural and Immunohistochemical Diagnosis of a Neonatal Herpes Simplex Virus Infection Presenting as Fulminant Hepatitis: A Case Report

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Abstract

TORCH (Toxoplasmosis, Rubella, Cytomegalovirus, Herpes Simplex Virus and Syphilis) infections are a major cause of intrauterine and perinatal infections with associated morbidity and mortality. Neonatal Herpes Simplex Virus infection caused by an enveloped, double-stranded DNA virus of the Herpesviridae family is devastating and fatal. Herpes Viruses are not hepatotropic but may rarely cause hepatitis. Most cases of HSV hepatitis rapidly progress to fulminant hepatic failure and often fatal before the diagnosis or transplantation. Nowadays, despite the availability of antiviral

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treatment (acyclovir), the outcome remains poor because of late identification of hepatic Herpes Simplex Virus (HSV) infection. We report a male neonate suspected with a metabolic/mitochondrial disease and multi-organ involvement but who developed a fulminant hepatic failure and disseminated coagulopathy secondary to HSV type 1 (HSV-1) infection. The postmortem diagnosis was performed demonstrating HSV-1 in liver tissue by transmission electron microscopy and by retrospective detection of HSV specific antigens by immunohistochemistry.

Keywords

Electron microscopy · Hepatitis · Herpes simplex · Postmorten diagnosis · Virus

1 Introduction

In the first 28 days-of-live intrauterine and perinatal infections are the major causes of neonatal death (Lehtonen et al. 2017). Neonatal Herpes Simplex Virus (HSV) infection is uncommon, deadly and caused by an enveloped virus of the Herpesviridae family (Basinger et al. 2019; Curry et al. 2006; Goldsmith 2014; Laue 2010; Motorna et al. 2018; Newcomb et al. 2017; Ramgopal et al. 2020; Roingeard 2008; Roingeard et al. 2019). HSV type 1 (HSV-1) causes 25–30% of the neonatal herpetic infections and leads to devastating diseases with potential poor outcomes. Infants acquire viruses during intrauterine, peripartum or postpartum periods, from a parent or caregivers. Neonates with HSV disease are classified in three groups: skin-eye-mouth, central nervous system (CNS), and disseminated disease. This classification is clinically relevant because it is predictive of neurodevelopmental outcome and mortality of the disease. Herpes Viruses, unlike Hepatitis Viruses, are not hepatotropic but may cause hepatitis, even if rarely (Noor et al. 2018). Most cases of HSV hepatitis rapidly progress to fulminant hepatic failure which can be fatal before both diagnosis and transplantation. Nowdays, although the pathogenesis of HSV hepatitis is not fully understood, it has high mortality rate and severe complications (Basinger et al. 2019; Noor et al. 2018; Then et al. 2019; Tosone et al. 2018). Clinical suspicion for neonatal HSV infection warrants immediate initiation of appropriate antiviral therapy with specific agents that dramatically improve clinical outcomes with manageable toxicity. For babies with disseminated and CNS disease, death is usually due to severe coagulopathy and extensive hepatic/pulmonary involvement despite the availability of therapy (McPherson 2020). This makes the early identification of HSV infection essential in improving outcomes and potentially preventing mortality (Then et al. 2019). We report a male neonate suspected with a metabolic/mitochondrial disease and multi-organ involvement who developed a fulminant hepatic failure and disseminated coagulopathy secondary to HSV-1 infection. The diagnosis was made postmortem demonstrating HSV in liver tissue by transmission electron microscopy (TEM) and detecting retrospectively HSV-1 specific antigens by immunohistochemistry (IHC).

2 Case Presentation

A male patient was born at term from dystocic delivery with the aid of vacuum extractor. Maternal pregnancy was normal with negative maternal serology (Cytomegalovirus, Toxoplasma gondii, HIV, Treponema pallidum, Rubella Virus, Hepatitis C Virus) and vagino-rectal swab (Streptococcus Agalactiae). He presented transient newborn respiratory distress responsive to nasal Continuous Positive Airway Pressure (nCPAP), a mild jaundice and a normal neonatal screening (Table 1) with negative Guthrie test, so he was discharged. On the third day of life, he was hospitalized at the Neonatology Unit (NU) of the birth point, due to hyperbilirubinemia (no laboratory data) and treated, until resolution, with phototherapy for 28 h. He was discharged on the fourth day of life in stable conditions and breastfed.

On the tenth day of life he was readmitted to hospital because of irritability, axial hypotonia, cyanosis and focal seizure activity and exposed to a broad-spectrum antibiotic therapy. In a few hours,

Table 1 Neonatal screening tests routinely done in the Neonatology and Neonatal Intensive Care Unit in S. Anna Hospital, Ferrara, Italy

Test	Result
Phenylketonuria	Negative
Hyperphenylalaninemia	Negative
Deficient biosynthesis of the biopterin cofactor	Negative
Deficient regeneration of the biopterin cofactor	Negative
Tyrosinemia type I and II	Negative
Maple syrup urine disease	Negative
Homocystinuria	Negative
Citrullinemia	Negative
Arginase deficiency	Negative
Argininosuccinate lyase deficiency	Negative
Carnitine transporter deficiency	Negative
Carnitine palmitoyltransferase I deficiency	Negative
Carnitine palmitoyltransferase II deficiency	Negative
Carnitine- acylcarnitine translocase	Negative
Very long chain acyl CoA dehydrogenase deficiency	Negative
Long chain 3 hydroxy acylCoA dehydrogenase and mitochondrial trifunctional protein deficiencies	Negative
Mediun chain acyl Coa dehydrogenase deficiency	Negative
Short chain 3 hydroxy acylCoA dehydrogenase deficiency	Negative
Glutaric aciduria type I	Negative
Glutaric aciduria type II	Negative
Isovaleric aciduria	Negative
Betaketothiolase deficiency	Negative
Propionic aciduria	Negative
Methylmalonic aciduria	Negative
Methylmalonic aciduria with Homocystinuria	Negative
3Hydroxy 3 methyl glutaric aciduria	Negative
2 Methylbutirril CoA dehydrogenase deficiency	Negative
Multiple carboxilase deficiency	Negative
Galactosemia	Negative
Biotinidase deficiency	Negative
Congenital adrenal hyperplasia	Negative
Congenital hypothyroidism	Negative
Cystic fibrosis	Negative

his clinical conditions dramatically worsened with impaired respiratory and cardiac function and oliguria. Laboratory tests showed: metabolic acidosis, coagulopathy, hyperammonemia, hypertransaminasemia, ketotic hypoglycemia (Table 2). The patient was transferred at our Pediatric Intensive Care Unit (PICU) where he arrived in profoundly serious conditions (Table 2); the PEdiatric Logistic Organ Dysfunction (PELOD) score was critical equal to 22.7. Supportive care was immediately started with metabolic correction measures, mechanical ventilation, inotrope (VIS first 24 h = 60), peritoneal dialysis after insertion of Tenckhoff catheter and blood products transfusion. Plasma amino acidogram showed a generalized elevation of all amino acids and plasma acylcarnitines determination showed minimal elevation of C2-C4-C8-C10. Hepatotropic virus (Epstein Barr Virus, Parvovirus, Cytomegalovirus) serology was negative. However, all treatments proved ineffective and the patient died 48 h after admission. Muscle and skin biopsies and DNA sample were taken in perimortem, with informed consent from parents, as usual in children with metabolic disease with not defined etiology. Autopsy was then performed.

Table 2 Laboratory data from the tenth to the twelfth day of life of the patient

	Age of life			
Exam	10th day (NU, Ferrara)	10th day (PICU, Bologna)	11th day	12th day
AST (<60 U/L)	–	7,662	4,647	–
ALT (<45 U/L)	2,714	1,254	729	–
PT (<1.20 s)	Sample does not coagulate	Sample coagulated	5.90	Hemolysis
A PTT (0.82–1.25 s)	Sample does not coagulate	Sample coagulated	7.8	Hemolysis
pH	–	7.15	7.14	6.91
LACTATE (0.5–1.4 mmol/L)	–	>15	>15	>15
BE (48 mmol/L)	–	−19.2	−13.8	−24.3
AMMONIA (<53 μmoli/L)	621	335	248	–
PCR (<0.50 mg/dl)	0.29	0.39	0.19	–
GLUCOSE (45–126 mg/dl)	<10 (ketons in urine)	–	–	–

ALT alanine aminotransferase, *AST* aspartate aminotransferase, *PT* prothrombin time, *APTT* partial thromboplastin time activated, *BE* base excess, *PCR* C-reactive protein

2.1 Muscle Biopsy

Muscle biopsy performed 1 day after hospitalization was snap-frozen in liquid nitrogen-chilled isopentane. Cryostat-cut sections were routinely stained for routine histology and histoenzimology and small fragments of muscle biopsy were fixed and embedded in araldite for analysis under TEM, as previously described (Cenacchi et al. 2013). Histological and histochemical stainings showed myopathic, aspecific findings such as fiber size variability, rare hypertrophic eosinophilic fibers, and slightly increased staining in the reaction for lysosomal acid phosphatase. Ultrastructural study showed few alterations of the myofibrillar component with disarray and mild increase of lipids with no marked evidence of mitochondria alterations. The histological and ultrastructural findings didn't confirm a diagnosis of mitochondrial or metabolic disease.

2.2 Autopsy

On autopsy, gross examination revealed multi organ failure with anasarca, pulmonary edema, bilateral cortical kidney ischemia, bilateral adrenal sub-atrophy and diffuse liver damage evidenced by small white areas in a red background, suggestive of necrosis caused by infection and/or shock. The cause of hepatic damage was not obvious at the time, but it was thought to be the initial cause of a sequence of damaging events. As routinely done, a thorough sampling of the internal organs was performed for histological study. Liver samples were submitted also to ultrastructural analysis (UA) because of the clinical suspicion of mitochondrial or metabolic disease and pathological suspicion of infectious disease. Moreover, hemorrhagic subcutaneous suffusions were suspicious for hepatic insufficiency and/or intravascular coagulopathy. Small gastric ulcers associated with hemorrhagic punctuations of the mucosa and mild splenomegaly were also described. Brain examination was not performed for ethical reasons.

2.3 Liver and Adrenal Gland Histology Analysis

Autoptic liver and adrenal gland biopsies were formalin fixed, paraffin embedded and stained with HE which showed a severe necrotizing hepatitis with confluent hemorrhagic necrosis with few areas of coagulation necrosis (Fig. 1a, b). Hepatic hemorrhagic necrosis is also a consequence of the shock associated with disseminated intravascular coagulopathy. Adrenal gland showed small areas of coagulation necrosis (Fig. 1c, d), that might have contributed to organ failure shock.

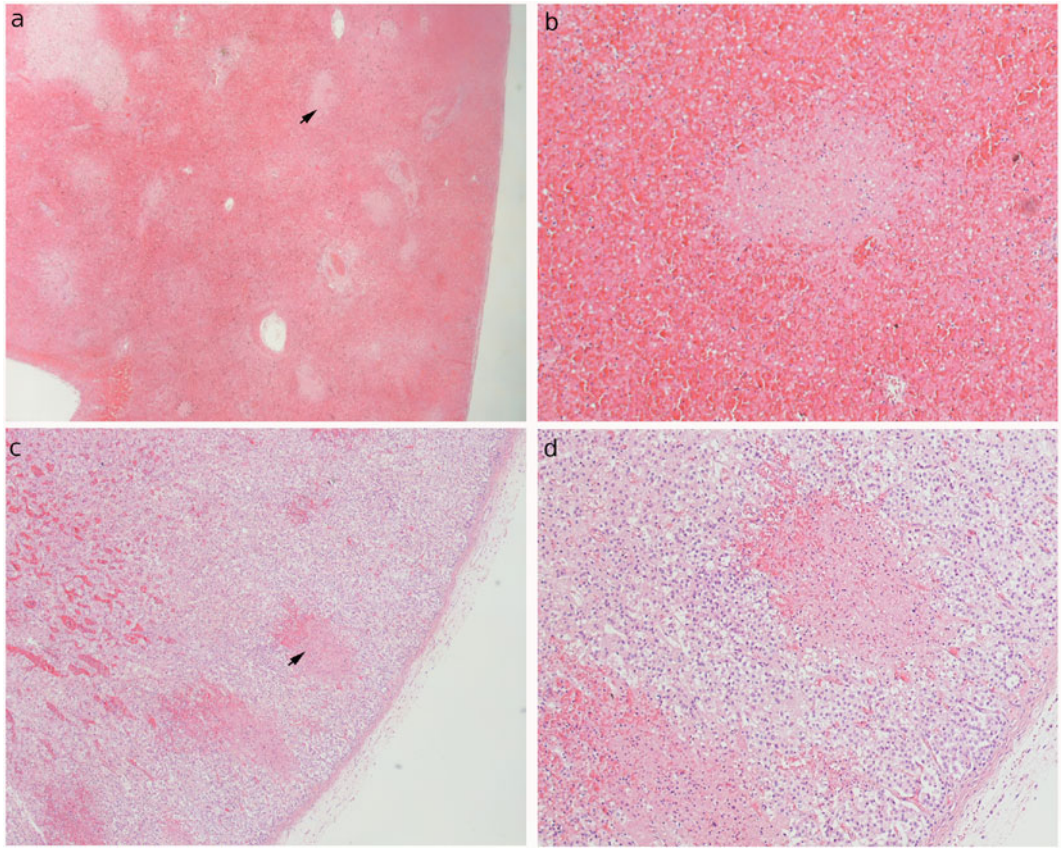

Fig. 1 Liver and adrenal gland autoptic samples, histology analysis. HE shows several areas of coagulation necrosis (arrows) in liver (**a**, **b**) and adrenal gland (**c**, **d**); magnification 2x (**a**, **c**) and 20x (**b**, **d**)

2.4 Liver Ultrastructural Analysis

As previously described small liver fragments were processed for UA, which showed hepatocytes with intracytoplasmic large lipid vacuoles (Fig. 2a) and irregularly shaped nuclei, despite artefactual post-mortem alterations (Cenacchi et al. 2013). Moreover, nucleoplasm and cytoplasm featured many roundish or polygonal nucleocapsid particles, 70–80 nm in diameter with an electron dense core (Fig. 2b). These particles were also found near the nuclear membrane frequently empty (defective particles) (Fig. 2c). In the intercellular areas some viral nucleocapsids were seen showing an envelope with an overall diameter of about 200 nm (Fig. 2d).

2.5 Liver and Adrenal Gland Immunohistochemical Analysis

After the result of UA, IHC was performed using a cocktail of anti-HSV antibodies composed by a rabbit anti-HSV-1 and a mouse anti-HSV-2; this cocktail reacts with both HSV-1 and HSV-2 specific antigens and it recognizes all the major glycoproteins in the viral envelope and at least one core protein (PDRM001, Diagnostic Biosystem). Using different secondary antibodies, respectively anti-rabbit and anti-mouse separately on different slides, we were able to identify nuclei and some cytoplasmic areas positive for HSV-1 and negative for HSV-2 both in liver (Fig. 2e) and adrenal gland (Fig. 2f). The overall histological

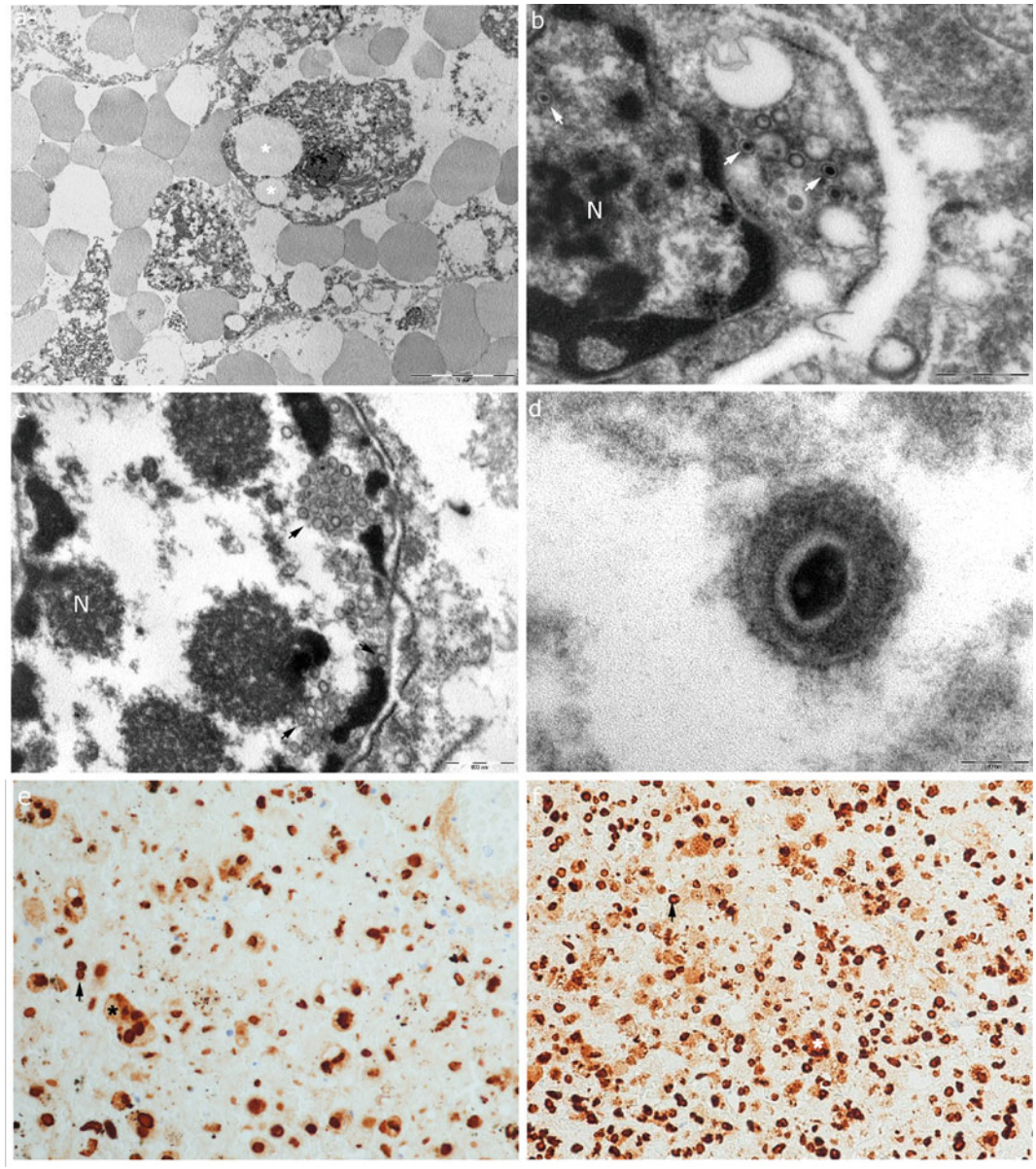

Fig. 2 Liver and adrenal samples, ultrastructural and IHC analysis. (**a**) Remnants hepatocytes with cytoplasmic lipid vacuoles (asterisks). (**b**) Nucleocapsidic particles with electrondense core are visible in the nuclei and cytoplasm (arrows). (**c**) Some empty defective particles are found alongside the nuclear membrane (arrows). (**d**) In the intercellular space, some virions are visible with an envelope of intermediate electrondensity, giving an overall diameter of 200 nm. Numerous HSV-1 positive nuclei in hepatic (**e**, arrow) and adrenal (**f**, arrow) tissues and few cytoplasmic areas (asterisks) by IHC analysis. Magnification 5,400x (**a**), 25,000x (**b**, **c**), 64,000x (**d**), 40X (**e**, **f**)

findings suggest an hematogenous dissemination of the HSV infection.

3 Discussion

Neonatal herpetic infections should be considered in the differential diagnosis of each acutely unwell neonate. Moreover, disseminated HSV infection should always be considered as a possibility, especially in neonates with CNS, hepatic, pulmonary or multiorgan disease processes. Early diagnosis is imperative when evaluating neonatal HSV infection to prevent further disease progression, neurological complications and even death. Even though treatment (acyclovir) is readily available, most cases have a poor prognosis due to late therapeutical initiation because of an infection misdiagnosis (Abuhasna et al. 2012). The differential diagnoses to consider in disseminated disease patients include other viral causes and metabolic/mitochondrial diseases. HSV infection might be suspected even though its presentation is frequently non-specific, difficult to be distinguished from bacterial sepsis and the maternal infection can be asymptomatic. Diagnostic tools include cultures of vesicular lesions and 'surface swabs', HSV serology, HSV DNA by PCR and liver biopsy histological examination. The latter remains the gold standard for diagnosing HSV hepatitis even if there is an increased associated risk of bleeding in patients with acute liver failure, sometimes reduced by a transjugular approach instead of a percutaneous one (Noor et al. 2018; Then et al. 2019). In the literature, this very rare diagnosis is frequently made through post-mortem biopsy, confirming that HSV hepatitis is usually characterized by a non-specific presentation, making timely diagnosis difficult and fatally delaying the following treatment. In our case, we have considered but excluded neonatal sepsis due to repeatedly negative C-reactive protein (Table 2) and supposed a potential mitochondrial/metabolic disease because of the early onset of symptoms, elevated ammonia and lactate as well as metabolic acidosis and ketotic hypoglycemia found already from onset. Histological and ultrastructural findings of the muscle biopsy did not confirm this hypothesis. We tried to investigate primary hepatic diseases, also infective ones (Epstein Barr, Parvovirus, Cytomegalovirus) but they were all negative. The extremely serious conditions of the child made difficult even recovering blood samples on which perform further analysis and the research of HSV DNA, which was possible only on DNA isolated from Guthrie test, was negative as well, excluding a peripartum maternal infection. We managed to carry out the plasma amino acidogram, which showed nonspecific findings, probably influenced by severe hemolysis, and the determination of plasma acylcarntins which did not justify the overall clinical picture. These no specific results couldn't guide appropriate genetic tests. The UA of the liver biopsy suspected the presence of enveloped viral particles compatible with Herpes Virus. Herpes and Cytomegalovirus share a similar ultrastructural morphology and the differential diagnosis has been done by IHC analysis. In addition, in the histology findings of the liver biopsy were absent cytomegaly and enlarged nuclei with the "Owl's eye" inclusions, typical of a Cytomegalovirus infection. IHC on liver sections identified HSV-1 as the causative agent, confirming the ultrastructural suspicion and reversing the initial metabolic hypothesis, which would have been negative for the parental perspective of future sons. In conclusion, we would like to aid clinicians to early identify of HSV hepatitis to rapidly initiate treatment. The antiviral therapy prevent mortality even if we cannot forget that liver transplantation may be the only option for neonates with fulminant or acyclovir resistant HSV hepatitis (Noor et al. 2018; Shahani 2016; Then et al. 2019; Vincenzi et al. 2017). Moreover, we would like to highlight the key role of the UA in viral infection diagnosis also in autoptic samples.

Declarations

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Ethics Approval Written informed consent for muscle and skin biopsies and autopsy were obtained from the patient's parents.

Availability of Data and Material Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Code Availability Not applicable.

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Antimicrobial Activity of Xibornol and a Xibornol-Based Formulation Against Gram-Positive Pathogens of the Respiratory Tract

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Abstract

Xibornol is known since the 70s and a xibornol-based formulation is commercialized as spray suspension for the antisepsis of the oral cavity and as adjuvant in pharyngeal infections caused by Gram-positive microorganisms. Herein, we evaluated the antimicrobial activity of xibornol and the xibornol-based formulation against common pathogens of the upper and lower respiratory tract.

Our results indicate that xibornol alone and the xibornol-based formulation have strong antibacterial action against *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *Staphyloccus aureus,* as well as against the two emerging pathogens *Actinomyces israelii* and *Corynebacterium ulcerans*. These findings highlight the antimicrobial potential of these drugs in the topical control of pathogenic Gram-positive bacteria of the respiratory tract.

Keywords

Antimicrobial activity · Bornilene · Topical drug · Upper respiratory tract · Xibornol

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1 Introduction

The normal microbiota of the human throat is a complex mix of hundreds of bacterial species. It includes streptococci, lactobacilli, staphylococci, corynebacteria, and actinomyces. In some conditions, most of the microorganisms that normally reside in the upper respiratory tract may be responsible for mild to severe infections, not only limited to mouth cavity or teeth (Bosch et al. 2013). Bacterial infections of the throat are very

common and are mainly caused by Group A β-haemolytic streptococci such as *Streptococcus pyogenes* (Oliver et al. 2018). Infections are generally limited to sore pharyngitis, but complications are frequent and include both suppurative (i.e otitis media, cervical lymphadenitis, mastoiditis, sinusitis) and non-suppurative (i.e. acute rheumatic fever, acute glomerulonephritis) diseases. Despite with less frequency, upper respiratory tract infections are also caused by the emerging pathogen *Corynebacterium ulcerans* (Hacker et al. 2016), which is occasionally responsible for tonsillitis, pharyngitis, sinusitis, and pneumonia (Otsuji et al. 2017).

The oral cavity is an important source of bacteria able to cause infections of the lungs and other body districts. In fact, microorganisms that cause community-acquired pneumonia (i.e *Streptococcus pneumoniae* and *Actinomyces spp.*) normally reside in the oropharynx. *S. pneumoniae* can colonize the respiratory tract of asymptomatic healthy carriers and spread from this area becoming responsible for a plethora of severe infections, which include pneumonia, meningitis, bronchitis, rhinitis, sinusitis, and otitis media (Paju and Scannapieco 2007). *Actinomyces israelli* is a normal constituent of the human microbiota. From the mouth, it can occasionally spread and produce actinomycosis, a severe and chronic invasive disease (Valour et al. 2014). Actinomycosis includes infections of odontogenic origin that mainly concern the perimandibular region, as well as infections of the respiratory tract (pneumonia, bronchitis and laryngitis) and other body sites. *S. aureus* is another important human pathogen long described as a constituent of the human microbiota of the upper respiratory tract, despite its role in the oral health and disease is still debated (McCormack et al. 2015). Due to the impact of the emergence of methicillin-resistant *S. aureus* (MRSA) strains, the control of this microorganism in the oral cavity is some concern, particularly in subjects undergoing oral surgery or hospitalization.

Xibornol (6-isobornyl-3-4 xylenol; Fig. 1) is an antiseptic drug for topical use. The drug is a phenolic derivative of bornan and shows strong lipophilic nature, making its water solubility very poor. Although xibornol was discovered in the late '60s (Capponi 1969), only few data reported in the '80s are available describing its effectiveness and very few recent data are available on this drug. The antimicrobial activity of xibornol was demonstrated against *S. aureus* and other Gram-positive bacteria, viruses and fungi (Morandini et al. 1985; Combe et al. 1988; Scaglione 2009; Verani et al. 2017; Fabbri et al. 1988). Detailed mechanism of action this drug remains unknown, but bacteria exposed to xibornol present a marked reduction in cellular division and in the synthesis of nucleic acids, proteins and peptidoglycans (Combe et al. 1988). Several studies demonstrated the therapeutic efficacy of xibornol in treating upper respiratory tract infections (Morandini et al. 1985; Olivieri et al. 1984) and the good tolerability of this drug (Morandini et al. 1985; Scaglione et al. 1988).

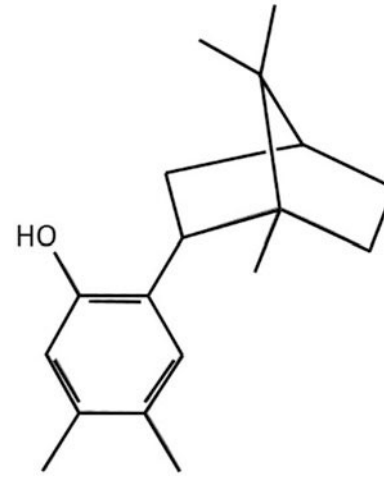

Fig. 1 6-isobornyl-3-4 xylenol (Xibornol)

A xibornol-based formulation is currently available on the Italian market sold as Bornilene (Agenzia Italiana del Farmaco – AIFA 2016). Bornilene is a complex spray drug that contains 3% xibornol and several excipients that facilitate spray dispersion and implement mucus adhesivity of the drug in the oral cavity (Scaglione 2009). Bornilene is sold for the topical treatment of infections and inflammatory states of the throat (pharyngitis, laryngitis, acute and chronic tonsillitis), for sustaining the hygiene of the mouth in

pre- and post-surgery, and as antiseptic in dental practice. With the exception of information on Bornilene reported on an AIFA document (AIFA 2016), there are no published data reporting the antimicrobial activity of this formulation. The aim of this work was evaluate the antimicrobial activity of xibornol and Bornilene against a panel of Gram-positive microorganisms that can cause infections of the respiratory tract.

2 Methods

2.1 Drug Formulations

Xibornol powder, Bornilene, and a placebo formulation were kindly provided by Abiogen Pharma (Abiogen Pharma S.p.a. Pisa, Italy). Bornilene and the placebo solution were contained in 30 ml bottles of plastic glass equipped with a pre-dosed metric valve and dispenser spout. Bornilene spray is a mixture of 3% xibornol and contains various excipient (glicerol, 96% (v/v) ethanol, micro-crystalline cellulose and 30% carmellose sodium, simeticon emulsion, clathrate menthol, ammonium glycyrrhizinate, hemihidrate chlorine buthanol, sodium saccharin, citral clathrate, anhydrous colloidal silica, castor oil, polyethoxylate, potassium). The placebo solution contained the solution of excipients of Bornilene but did not contain xibornol. Bornilene and the placebo solution were nebulized in a sterile tube, using the provided nebulizing bottle. 12% (w/v) solutions of xibornol were prepared in DMSO. Nebulized compounds and xibornol solutions were used immediately.

2.2 Bacterial Strains and Culture Media

Streptococcus pneumoniae NCTC 7465 (corresponding to ATCC 33400), *Streptococcus pyogenes* ATCC 12344, *Corynebacterium ulcerans* ATCC 51799, *Staphylococcus aureus* DSM 799 (corresponding to ATCC 6538), and *Actinomyces israelii* ATCC 12102 were used. *S. aureus* was propagated in TSA (Triptone Soy Agar; Oxoid, Basingstoke, UK), *S. pyogenes* and *S. pneumoniae* were propagated in TSA supplemented with 5% defibrinated horse blood, *C. ulcerans* and *A. israelii* were propagated on BHIA (Brain Heart Infusion Agar; Biolife, Milan, Italy). Cultures were incubated at 37 °C in aerobic condition (*S. aureus*, *C. ulcerans*), or 5% CO2 (*S. pyogenes* and *S. pneumoniae*), or in anaerobiosis (*A. israelii*).

2.3 MIC and MBC Determination

Minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) were determined in order to assess antimicrobial activity of the three different formulations (xibornol solution, Bornilene, placebo). Microdilution assays were performed according to the European Committee on Antimicrobial Susceptibility Testing standards (EUCAST 2017) in a 200 µl volume/well using, in the first well, 100 µl of the formulations prepared as described above. Mueller Hinton (MH; Oxoid) and Mueller Hinton-Fastidious (MH-F, Mueller Hinton supplemented with 5% defibrinated horse blood and 20 mg/L β-NAD; Oxoid) were used for conventional and fastidious microorganisms respectively. Incubation was performed at 37 °C in aerobiosis, 5% CO2, or anaerobiosis depending on microorganisms. Bacterial colonies were suspended in sterile 0.9% NaCl solution to 0.5 McFarland, a 1:10 dilution was performed and 10 µl (about 5 x 10^4 CFUs) used to inoculate microplate wells. For each assay, two consecutive plates (containing 24 dilutions each) were prepared. Due to the low water solubility of xibornol, the drug has been used starting from a 3% (w/v) concentration from a 12% stock solution in DMSO. The Bornilene and placebo solutions were used as supplied starting from a 1:2 dilution in the first well. Non-inoculated medium (C-), medium alone inoculated with bacteria (C+), DMSO dilutions (the same as xibornol) were used as controls for each assay. Assays were performed in triplicate. MIC was determined following the EUCAST reading guide (EUCAST 2017) and by seeding

appropriate dilutions of each well and counting CFUs after incubation at 37 °C for 24 h. MBC were calculated by seeding appropriate dilutions of aliquots of suspensions deriving from the wells corresponding to the MIC and higher drug concentrations and by CFU counting after incubation in the appropriate culture medium.

3 Results and Discussion

The oropharyngeal cavity is normally colonized by a wide variety of microorganisms that constitute a complex microbiome. From this district, pathogens may spread and cause local or systemic diseases (Avila et al. 2009). Although antibiotics are largely used worldwide to treat and prevent infections that originate from the upper respiratory tract, development of bacterial resistance is of great concerns. For this reason, the use of alternative or additional medications, such as oral antiseptics, should be strongly encouraged in view to control pathogen spreading, as well as dental (Slots 2002) and oral health (Shi et al. 2013).

Several antiseptics are available to control potential pathogenic microorganisms residing in the upper respiratory tract and include the widely used chlorhexidine (Karpiński and Szkaradkiewicz 2015) and povidone-iodine (Kanagalingam et al. 2015). Other antiseptics, such as octenidine (Assadian 2016), polyhexanide (Fjeld and Lingaas 2016), hexetidine (Kapić et al. 2002), and xibornol (Scaglione 2009) are also available.

Xibornol has long history of safe use as adjuvant and antiseptic and some studies regarding xibornol activity against bacteria do exist (Morandini et al. 1985; Combe et al. 1988; Scaglione 2009). However, the strong lipophilic nature that affects handling of xibornol in antimicrobial dilution assays probably contributed to limit the studies performed on this compound. Xibornol is commercially available as Bornilene, a well-tolerated drug containing 30 mg/ml xibornol (6-isobornyl-3-4-xylenol) that is used for topical administration in the oral cavity and the upper respiratory tract (AIFA 2016). To make xibornol more usable for topic action in the upper respiratory tract, a complex mixture of excipients was used in the formulation Bornilene.

In this work, we choose a panel of Gram-positive microorganisms that can be harmful for the human respiratory tract. In addition to the most famous *S. pneumoniae*, *S. pyogenes*, and *S. aureus*, we included the emerging pathogens *C. ulcerans* and *A. israelii*, which have an established role in causing respiratory and extra-respiratory infections. The activity of xibornol, Bornilene, and the placebo solution contained in Bornilene was determined by microdilution assays. As shown in Table 1, results indicate that the MIC values of xibornol against the tested bacteria ranged from 0.003% for *S. aureus* and the two streptococci, to a concentration of 0.0005% for *A. israelii* that resulted the most sensitive among the considered microorganisms. Xibornol showed bactericidal action against *S. aureus*, *A. israelii*, and *C. ulcerans* (MBC: 0.003%, 0.0005%, 0.001%, respectively), being the MIC and MBC values identical for these organisms. For *S. pyogenes* and *S. pneumoniae*, MBC values (0.012%) were two dilutions higher than MICs, indicating that at the MIC values the drug had bacteriostatic effect against these two pathogens. As expected, similar results were obtained with Bornilene. The MIC values of this complex formulation ranged from 1:1024 (0.0029% xibornol) for *S. pyogenes* to 1:4096 (0.0007% xibornol) for *S. aureus* and *C. ulcerans*. Similar to xibornol, Bornilene showed bactericidal effect against *S. aureus* (MBC 1:4096), *A. israelii* (MBC 1:2560) and *C. ulcerans* (MBC 1:4096). MIC values against *S. pneumoniae* and *S. pyogenes* (1:20148 and 1.1024, respectively) were lower than MBC values, thus indicating that, similar to xibornol, at the MIC values Bornilene has bacteriostatic effect on these organisms. The placebo solution had no activity against all the tested microorganisms.

In the case of *S. pneumoniae* and *S. aureus*, MIC and MBC values of Bornilene were slightly lower than those of xibornol alone (Table 1). This effect could result from the presence of the excipients contained in Bornilene. Although excipients alone do not show antimicrobial

Table 1 MIC and MBC values of xibornol, Bornilene, and placebo against Gram-positive microorganisms

	xibornol		Bornilene		placebo	
Bacterial species	MIC	MBC	MIC (% xibornol)	MBC (% xibornol)	MIC	MBC
S. pneumoniae	0.003%	0.012%	1:2048 (0.0015)	1:1024 (0.0029)	n	n
S. pyogenes	0.003%	0.012%	1:1024 (0.0029)	1:512 (0.0058)	n	n
S. aureus	0.003%	0.003%	1:4096 (0.0007)	1:4096 (0.0007)	n	n
A. israelii	0.0005%	0.0005%	1:2560 (0.0012)	1:2560 (0.0012)	n	n
C. ulcerans	0.001%	0.001%	1:4096 (0.0007)	1:4096 (0.0007)	n	n

n: no activity

activity, their presence in Bornilene could increase xibornol bioavailability and activity towards these organisms.

Bornilene is used without dilution by direct spraying it on tissues of the oral cavity and pharynges. Our finding that Bornilene is active at high dilutions suggests that this drug is very efficacious when administered *in vivo*. In addition, the presence of xibornol in a glycerol-based formulation, which displays strong mucoadhesion properties (Jones et al. 2007), most likely confers longer persistence of the drug on mucosal tissues, thus reducing its wash-out and prolonging its activity.

4 Conclusion

In the present paper, we evaluated MIC and MBC of xibornol and the highly water-insoluble xibornol-based mixture Bornilene against Gram-positive pathogens of the upper respiratory tract. We found that both the drug alone and the drug suspended in excipients have strong antibacterial action against *S. aureus*, *S. pneumoniae*, *S. pyogenes*, and the two emerging pathogens *C. ulcerans* and *A. israelii*.

Our findings indicate that xibornol-based formulations can be very effective in the topical control of pathogenic Gram-positive bacteria relevant in upper and lower respiratory tract, odontostomatologic, as well as systemic infections. Due to the dramatic increase of bacterial resistance to a number of commonly used antibacterial agents, xibornol-based formulations, such as Bornilene, appear as valid antiseptic for controlling and reducing bacterial colonization and infections also caused by antibiotic-resistant bacteria.

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Achille Sclavo (1861–1930) and His Innovative Contributions to Italian Preventive Medicine and Healthcare Policy

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Abstract

Achille Sclavo was a scientist with a multifaceted personality; throughout his life, he steadfastly maintained his commitment to research and teaching, while also gaining precious experience as an educator, politician and entrepreneur. He carried forward these various activities with the aim of bringing relief to a country smitten by epidemic diseases, of spreading the gospel of hygiene, and of creating and training medical and healthcare personnel that would be able to tackle the difficult problem of public health in Italy at the beginning of the twentieth century. In this regard, Achille Sclavo can undoubtedly be considered a precursor of modern public health and preventive medicine.

Keywords

Achille Sclavo · Health policies · Hygiene · Infectious diseases · Public health

1 Achille Sclavo's Family Background and Education

Achille Sclavo was born in Ceva (CN) on 23 March, 1861. His family originally came from Lesegno, a small village in the Province of Cuneo in Piedmont. He was actually baptized with the name Vincenzo, as is recorded in the archives of the University of Turin, where he was admitted to the School of Medicine in 1881 after completing his high-school studies at the College of the Scolopi Fathers in Savona.

Sclavo graduated in Medicine on 19 July, 1886, and immediately began in-service training at Cottolengo Hospital, where he specialized in pharmacology and dermo-syphilography.

After graduating, he married Eugenia Pertusio, with whom he had five children: Maria Teresa (1892), Rosalia (1894), Eugenio (1898), Piero (1900) and Matilde (1902).

The turning point in Sclavo's professional career came in 1887, when he was called to the General Directorate of Health in Rome by his mentor, Prof. Luigi Pagliani, as a member of the work-group founded by Francesco Crispi

The original version of this chapter was revised as the 3 figures were missed to be included to the text. This has now been corrected and the 3 figures have now been inserted to their correct positions in the text. A correction to this chapter can be found at https://doi.org/10.1007/5584_2022_714

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(Minister of the Interior) to reorganize national healthcare policy (Crispi–Pagliani Law, n° 5,849 of 22 December 1888, the first Italian healthcare reform). Here, he first served as medical secretary and then as assistant at the scientific laboratories, where he worked alongside Pagliani himself, Pietro Canalis and Alfonso di Vestea. "This was the beginning of that fervid friendship among the four Masters which never waned over the years, which was strengthened by their common determination to improve public health, and which was one of the chief mainstays of healthcare reform in our country" (Mazzetti 1949).

In 1892, Sclavo was appointed Director of the Bacteriology Laboratory and obtained a teaching post at the Scuola Superiore di Igiene (Upper School of Hygiene). During this period, he undertook original studies on anti-diphtheria and anti-anthrax sera.

Encouraged by the results of Louis Pasteur's research on the techniques of weakening the virulence of germs and on the consequent implications for the production of new vaccines (live attenuated vaccines), between 1895 and 1903 Sclavo embarked upon intensive experiments on the biology of *Bacillus Anthracis*, the sporiparous causative agent of anthrax, an infectious disease affecting shepherds, farmers and, especially, tanners; a zoonosis chiefly affecting ruminants, this disease also had a heavy economic impact on cattle and sheep farming (Caldwell et al. 2017; Mancini and Ippolito 2003; Holgate and Holman 1949; Hodgson 1941; Fergusson 1911; Lockwood and Andrewes 1905; Mitchell 1905).

His studies led to the introduction of a new method of eliciting immunity to anthrax in animals and humans, which involved the use of serum from infected sheep, rather than Pasteur's vaccine. Moreover, unlike the vaccine, this serum could also be used to treat the disease. Sclavo devoted many of his writings to this subject, from the first *Sulla preparazione del siero anticarbonchioso* (On the preparation of anti-anthrax serum) (1895), when he was still working at the General Directorate of Health in Rome, to the last *Intorno ad alcune proprietà del siero anticarbonchioso* (Concerning some properties of anti-anthrax serum) (1906), which was signed jointly with his pupil Donato Ottolenghi.

Fig. 1 Achille Sclavo (Elderly Group Archive, Sclavo, Siena)

During his directorship of the Bacteriology Laboratory, Sclavo was also involved in food science, conducting studies on the characteristics and chemical and physical properties of foodstuffs, with the aim of combating the adulteration of foodstuffs and ensuring their genuineness. More generally, he worked to improve hygiene in Italy by implementing the principles of microbiology. In the light of the insights provided by this new discipline, he tackled the 1893–94 cholera epidemics, particularly in the Provinces of Alessandria and Campobasso, and also studied the differences among the bacterial strains involved in these epidemics (Fig. 1).

2 Research and University Teaching

With the fall of Crispi's second government in 1896, the General Directorate of Health that had been created – the "forge of the first group of Italian hygienists" (Mazzetti 1949) – was suppressed by the Minister Di Rudinì. Many of the hygienists who had worked there, including

Sclavo, embarked upon an academic career, giving rise to a major modernization of medical faculties. After being appointed to teach public health in Turin in 1893, Sclavo was charged with teaching this discipline at the University of Siena for the academic year 1896–1897, a post that was also confirmed for the following year. In 1898, he moved to Sassari as an extraordinary professor of public health, but returned to Siena after only 6 months to take up a post as extraordinary professor of public health and director of the public health laboratory.

These were the beginnings of Sclavo's university career. Subsequently, with the royal decree of 29 March 1903, he was appointed on 1 April to the post of Ordinary Professor of Public Health at the University of Siena, and with the decree of 21 July 1907, became Dean of the School of Medicine and Surgery.

In 1915, Sclavo was called to Florence as the Director of the Military School of Health, where he organized 50 courses in bacteriology and anti-tuberculosis prophylaxis, and, in the following year, in bacteriology and disinfection. In 1917, he also lectured the female nursing staff of the Italian Red Cross on the prophylaxis of infectious diseases and on diet.

In his teaching, which essentially focused on bacteriology, disinfection and anti-tuberculosis prophylaxis, he was supported by a group of co-workers, among whom his pupil, the hygienist Giovanni Petragnani (Martini et al. 2018a).

In 1918, he was unanimously invited to teach public health at the School of Medicine in Florence, where he remained for 3 years, before returning to the University of Siena where he taught until his death.

In 1919, he received the title of Emeritus Professor (Ministero dell'Istruzione 1919). At the University of Siena, he also held the position of Rector three times: from 1914 to 1917, from 1924 to 1926, and from 1927 to 1929.

As further testimony to his scientific skills, in 1918 he was called to participate in the government commission charged with drawing up provisions concerning public health, which were necessary to the transition from wartime to peacetime (Fig. 2). In 1921, he founded the National Association of Hygienists, which considerably oriented future policies in healthcare, prophylaxis and social medicine.

Achille Sclavo died on 2 June 1930 in Genoa, where he had retired for health reasons.

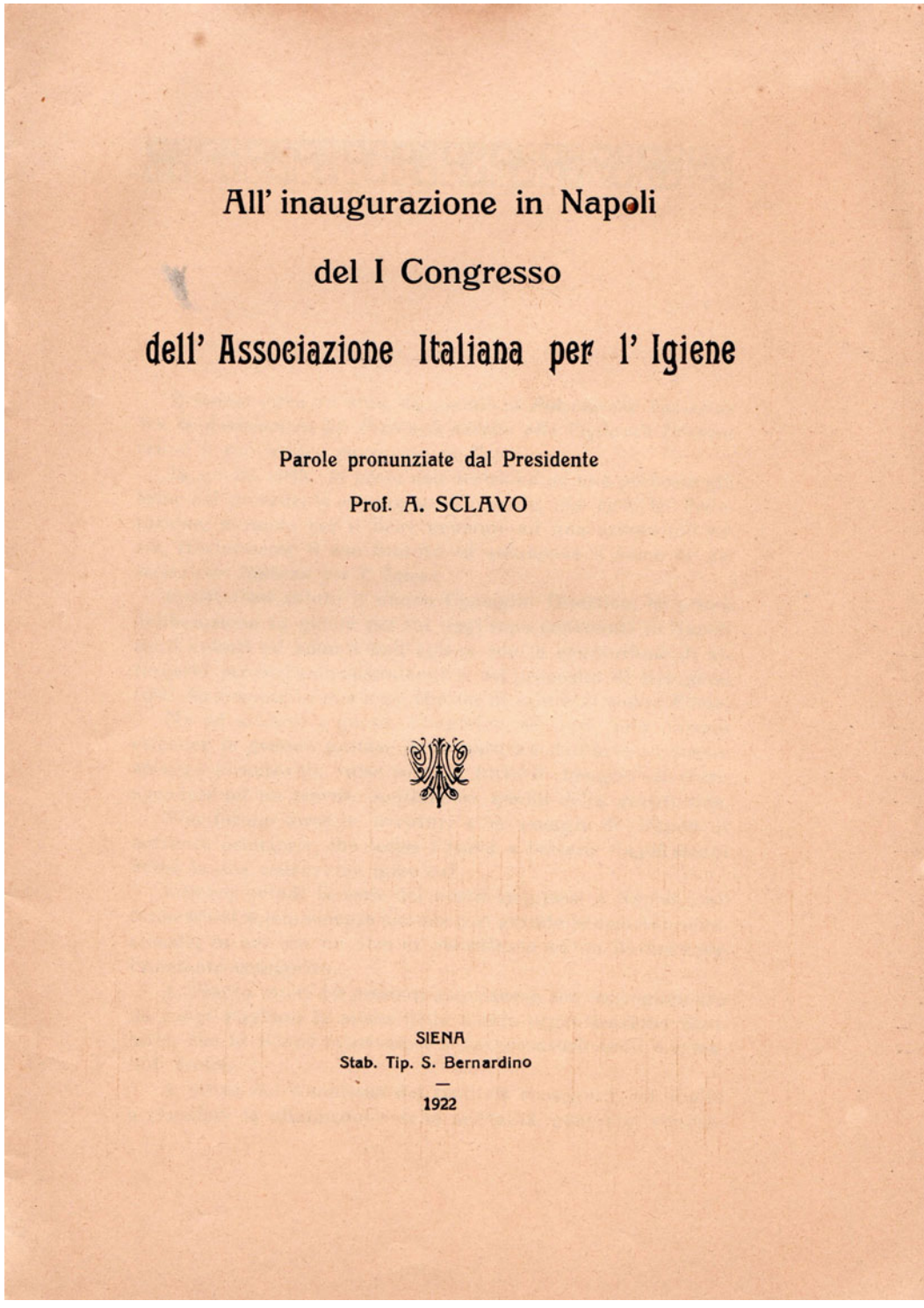
3 Sclavo's Contributions to National Healthcare Policies and to Education

Between 1893 and 1911, Sclavo was personally involved in healthcare policy campaigns and the fight against the serious diseases that afflicted many parts of Italy, where the health of the population was strongly conditioned by such factors as poverty and ignorance.

His efforts "in the field" were unceasing, and in 1893 he fought cholera epidemics in Alessandria and Campobasso. In Certaldo and Poggibonsi in Tuscany in 1905, he conducted the first Italian anti-typhoid vaccination campaigns using the vaccine created by Almroth E. Wright (1861–1847).

In 1910, together with the pathologist Alessandro Lustig (1856–1937), he headed Sardinia's first anti-malaria campaign in the Province of Sassari. This resulted not only in the establishment of local facilities for therapy and prophylaxis by means of state-supplied quinine, but also in environmental reclamation works and educational programs in hygiene. In the same year, he was a member of the ministerial commission for the study of pellagra. The following year saw him fighting a cholera outbreak in Puglia, where he stressed the importance of urban infrastructure, such as sewers (Sclavo 1912), and of isolating affected subjects. Finally, in 1911, he went to Palermo and the surrounding area to stamp out epidemics of smallpox and cholera.

In many parts of Italy, Sclavo promoted the use of civil engineering works to combat several epidemic diseases which, especially in summer, afflicted many Italian cities on account of their primitive conditions of hygiene. "*He adopted a new approach to everything, from the supply of safe drinking water to the activities of the municipal police. He enlisted the aid not only of*


All' inaugurazione in Napoli

del I Congresso

dell' Associazione Italiana per l' Igiene

Parole pronunziate dal Presidente

Prof. A. SCLAVO

SIENA

Stab. Tip. S. Bernardino

—

1922

Fig. 2 Speech of 1921 by Achille Sclavo at the 1st Congress of the Italian Association for Hygiene (Elderly Group Archive, Sclavo, Siena)

doctors, but also, and especially, of chemists and of veterinarians, whom he called upon promptly, perhaps believing that creating a hygiene mentality required this broader vision" (Ragazzi 1954). In all these situations, however, he had to combat not only the epidemics underway, but also prejudice, superstition and ignorance.

From the beginning of his career, Sclavo tackled the afflictions of childhood. In the opening speech of his university course in Siena in 1896, he maintained that childcare should be given first place in a healthcare program aimed at reducing infant mortality. He therefore advocated that orphanages be brought within the sphere of university teaching, in order to improve the training of doctors and caregivers (Sclavo 1922), and that a chair of pediatrics be instituted in every medical school, so that "*medical students might extend their competence to everything concerning the treatment and prevention of childhood diseases*" (Sclavo 1897).

In the following years, he increasingly emphasized the importance of focusing on childhood, both in order to improve children's health, and therefore reduce mortality in the first years of life, and as a primary objective of education aimed at promoting the population's knowledge of hygiene.

Hence, he became involved in the creation of school facilities that conformed with the principles of hygiene, and which in Siena took the form of a nursery and an open-air school built on the terraces of the Fortezza Medicea (Orsini 2020a). His aim was to create salubrious environments where the precepts of hygiene could be integrated with physical education, and where the principles of the fight against tuberculosis could also be implemented (Orsini 2020b; Martini et al. 2018b).

The difficult situation of schools was also a subject of his writings: *Igiene ed edilizia scolastica* (Hygiene and school buildings) (1914), in which he denounced the disconcerting conditions of school buildings and proposed solutions to the most widespread health problems.

In those and subsequent years, he also published various writings in which he prescribed a healthy diet, physical exercise and outdoor education for the healthy development of young people: *Per l'educazione fisica* (In favor of physical education) (1914); *Diamo aria ai nostri polmoni* (Giving our lungs air) (1915), the manifesto of the anti-tuberculosis campaign in favor of fresh air and sunshine; *Sull'alimentazione umana* (On human nutrition) - 5 lectures to the nurses of the Italian Red Cross in Florence (1917); and *Per l'igiene sociale* (Promoting social hygiene) (1918).

This commitment to healthcare campaigns, and more generally to solving widespread health problems in Italy at the time, earned him many official awards. More importantly, however, it brought him into direct contact with the people, enabling him to gain first-hand knowledge of their difficult living conditions and needs, especially the need for education.

Sclavo's daily dealings with epidemic diseases led him to realize that a great many cases could be avoided by following simple rules of hygiene and taking simple precautions to reduce the spread of infections.

> *All this, and many other considerations made on similar occasions, convinced him of the need to undertake hygiene propaganda, which constituted the main driving force of his activity as an educator* (Mazzetti 1949).

Indeed, he was deeply convinced that, alongside the work of researchers and doctors, a veritable "interior revolution" needed to be sparked within the population: a revolution that could foster the progress of public health and engender a healthy respect for human life in its psychophysical unity. To achieve this, as he declared at the 2nd Congress of the Italian Association for Public Health in Venice in 1923, it was necessary to "*Win over the soul of the people to the cause of Public Health*" (Sclavo 1923).

Thus, the educator Sclavo, who was among the first to hold university courses in Public Health at the Universities of Siena and Florence, and who, between 1915 and 1916, had been appointed Director of the Military School of Health in Florence (where he organized courses in bacteriology and tuberculosis prophylaxis for doctors and healthcare professionals), finally decided to address a broader audience: military personnel,

churchmen, general practitioners, functionaries in charge of public services, ordinary people… In this regard, he recalled "*My down-to-earth conversations continued, perhaps not fruitlessly, in front of a good number of highly attentive persons, even beyond my every expectation*" (Sclavo 1925).

> *Sclavo moved between two spheres, so to speak: that of higher education and that of teaching addressed to the people, this latter being much more difficult to implement, necessitating great powers of persuasion and, as in apostleship, faith in the truths proclaimed. It is in this shift that we find his leitmotif in the full maturity of his work. What he had observed in his life led him to regard youth as the most fertile ground in which to sow the precepts of hygiene, because […] like wax, youth adapts, but like bronze it retains the impression received* (Mazzetti 1949).

Very soon, however, Sclavo realized that a solid knowledge of hygiene could only be acquired through a slow process of learning, and that this was difficult to carry out in the domestic setting, owing to the ignorance and prejudice of parents. It therefore had to be implemented at school, by acting on teachers and, especially, pupils, who were free from the erroneous beliefs of adults and open to change. As he wrote, "*In all my efforts to protect against infectious diseases, I think I brought some innovation to the methods of propaganda by insisting particularly on spreading the word among elementary school children. In this way, I counted on what I often call ascending education, and with very good results*" (Sclavo 1954). This "ascending education" involved using children to carry the message of good practices to adults (parents and relatives), who, though obdurate towards such innovations, could recognize their importance and utility.

On this theme, it is particularly interesting to analyze the book that the great Sienese hygienist published in 1924: *Per la propaganda igienica. Scuola ed igiene* (In favor of hygiene propaganda. Schools and hygiene). The text was conceived in the country camp near Florence, which hosted the children of parents with tuberculosis, and was dedicated to "*the elementary school teachers, from whom Italy expects the most efficacious propaganda in favor of hygiene*" (Sclavo 1924), so that they might be able to fulfill their mission as educators of the young with respect to public health. Written in a deliberately simple style, in order to readily communicate the basic concepts of hygiene and medical sciences, the book is a veritable model of how to spread knowledge, and forcefully reveals the ingenuity of the scientist, the acuteness of the politician and the simplicity of the common man. From it, Sclavo emerges as a new figure: that of the doctor-hygienist who, in addition to his specialist knowledge, exhibits a deep awareness of the social and healthcare situation of his day, great humanitarianism and an extraordinary fervor in his lay apostleship.

Sclavo was convinced of the need not only to educate children and young people, but also to instill in them the awareness of hygiene. The novelty of his teaching, and perhaps also the reason why it was successful, lay in his ability to detach himself from traditional, standardized and arid teaching methods. Indeed, he claimed that "*First, the child should be provided with a salubrious, well-run school, where he should pick up those habits of cleanliness and hygiene that will protect him from many diseases. Moreover, his brain should be trained in the most appropriate manner, without excessive fatigue and with judicious choice of what it should take in, in order to foster cognition, conviction and will. School should educate the child to live in society […] As these tasks are very closely interrelated, it is advisable that the hygienist cooperate actively and cordially with the teacher, the one helping the other*" (Sclavo 1924).

The new school that Sclavo proposed therefore had new and broader educational objectives, including the development of a thoughtful, critical attitude. Indeed, on the basis of experience and the observation of phenomena, the pupils could play an active role in the educational process. In this setting, the role of the teacher, albeit still central, was markedly different. And this was the most innovative feature of Sclavo's educational work: what he proposed was not a lesson but a dialogue, in which the children were prompted to reason on scientific concepts, sometimes even complex ones. Indeed, while he never used the word "lesson", he constantly referred to

"conversation". This was a deliberate choice of terminology, which reflects Sclavo's rejection of a professorial style of teaching in favor of a new model of education based on the construction of knowledge.

4 The Tuscan Serotherapy and Vaccinology Institute Is Founded

Sclavo's studies on anthrax and the publication in 1903 of his paper *Sullo stato presente della sieroterapia anticarbonchiosa* (On the current state of anti-anthrax serotherapy) earned him the prestigious "Premio Riberi", which was awarded to him by the Academy of Medicine in Turin in the same year. On that occasion, Prof. Pagliani declared: *"It is only a profound faith in conscientious scientific research, illuminated by a superior culture and supported by very rare constancy and tenacity, that enabled Sclavo to triumph in his studies, despite the serious obstacles that he encountered at every turn. It is on account of this faith that our country owes him the honor of having placed a fecund milestone on the road to serotherapy treatments. Indeed, after Behring's anti-diphtheria serum, it is undoubtedly only Sclavo's anti-anthrax serum which, amid so many similar discoveries of an ephemeral and not always honored nature, remains superior to every objection and promises to be usefully applied in the treatment of a disease that afflicts both man and valuable livestock animals"* (Petraganini 1938).

With the funds from the Riberi Prize and the financial support and encouragement of his wife Eugenia, Sclavo bought the "villetta dell'ebreo", a villa on the outskirts of Siena. Here, on 3 November 1904, he and Ivo Bandi (1867–1926) founded the Istituto Sieroterapico e Vaccinogeno Toscano (Tuscan Serotherapy and Vaccinology Institute) " for the preparation of sera, vaccines, viruses, related products and therapeutic and prophylactic materials of other kinds" (Atto costitutivo dell'Istituto Sieroterapico e Vaccinogeno Toscano).

Ivo Bandi, graduated in Medicine in Pisa in 1892, in 1901 he accepted also the invitation of the Brazilian government and founded the *Bacteriological Institute of the State of São Paulo.*

After founded in Italy, in 1904 the Tuscan Serotherapic and Vaccinology Institute with Achille Sclavo, in 1910 he moved to Naples where in 1014 he founded the Italian Serovaccinotherapy Institute.

The Tuscan Serotherapic and Vaccinogen Institute was the beginning of an adventure that saw Sclavo – like other colleagues, including Serafino Belfanti who founded in 1896 the Milanese Serotherapic Institute, the first medical research institute dedicated to vaccines – don the garb of the entrepreneur.

Nevertheless, his primary objective was to contribute to the care and "hygienic redemption" of people. Aware that he could make a greater contribution to the country's battles in the field of hygiene and health care, Sclavo, in addition to his teaching and university research, created a center for the large-scale production of his anti-anthrax serum and of other sera and vaccines yielded by his and his co-workers' research.

The first two decades of the twentieth century saw a better and more diversified production of sera and vaccines. In healthcare policy aimed at avoiding or preventing the spread of infectious diseases, and in campaigns against so-called "social" diseases (owing to their severe impact on the population) serotherapy became essentially a curative technique, while vaccine therapy was used for the prevention of infectious diseases.

At that time, pharmaceutical production in Italy typically shifted from the small galenic laboratories of pharmacies to industrial facilities. Sclavo's Institute, by contrast, took a different path, dovetailing entrepreneurial activity and research. Especially in the initial years, Sclavo's priority remained research, which he also subsidized through the revenues on the sales of the Institute's first products.

A document in the archives of the Gruppo Anziani Sclavo (Sclavo Group of Elders) (Orsini 2016), dated 25 May 1907, enables us to reconstruct a list of the products of the Tuscan

Fig. 3 Printed advertisements Jodogelatina Sclavo, 1914 (Elderly Group Archive, Sclavo, Siena)

Serotherapy and Vaccinology Institute with extreme precision; it is a printed invoice listing all the pharmaceutical specialties available: anti-anthrax serum, anti-diphtheria serum, anti-typhoid vaccine and anti-anthrax vaccine. Another item mentioned is a medicinal specialty, "Jodogelatina Sclavo" (Fig. 3). "Discovered and introduced into therapy by Prof. Sclavo, it is a special combination in which iodine is incorporated into the molecule of gelatine"; it had the following broad (to say the least) therapeutic indications: "scrofula, rickets, arteriosclerosis, organic decay, chronic forms of malaria, tubercular forms, goiter, syphilis, gout" (Istituto Sieroterapico e Vaccinogeno Toscano 1934).

The scientific breakthroughs taking place in microbiology and immunology at the beginning of the twentieth century considerably boosted the production of the Institute, which began to expand its facilities. However, it was the First World War that prompted its greatest growth, with the industrial production of sera and vaccines, particularly against typhus and cholera, for military personnel. Indeed, the war provided a fundamental test-bench for the Institute and for the Italian pharmaceuticals industry in general. At the end of the war, attention was turned to the needs of the civilian population, which, exhausted and hungry as it was, fell victim to epidemics, most notably the tragic pandemic of Spanish influenza.

Finally, in the 1920s, the healthcare campaigns implemented by the fascist regime prompted a further increase in the production of the Institute, whose diphtheria and tetanus anatoxins (Anadifterall and Anatetall) practically stabilized the immune status acquired by the subjects vaccinated.

Moreover, with regard to sera and vaccines, it is interesting that, at least until the death of its founder, the Institute had a privileged relationship with the State, which was its chief customer. This was intentional, as Sclavo always tried to bring together the country's best resources in the effort to improve national healthcare: the University, which supported the Institute's scientific research; the ministries responsible for the various propaganda campaigns to fight epidemic

diseases and to affirm the basic rules of hygiene; the Institute and the city of Siena itself, where for decades the enterprise founded by Sclavo was one of the few business activities, if not the only one. And all of this was in line with the ethical sense of the State that had always inspired Sclavo, and which he always wanted to maintain, not least through a policy of low prices that enabled even the poorest strata of the population to purchase his products (Vannozzi 2005).

When the company was sold to private investors at the end of the twentieth century, the Sclavo trademark was withdrawn and donated to the Municipality of Siena. For some years now, as a tribute to Sclavo's ideals, it has been used by the non-profit Achille Sclavo Foundation, the aim of which is to accelerate the development of new vaccines and to make them available to poor countries at a sustainable cost, in order to fight infectious diseases and the poverty that they cause.

5 Conclusions

To fully appreciate the work of Achille Sclavo, we need to understand that it was always oriented towards a dual objective: scientific and social.

> *We owe much to Achille Sclavo and to the colleagues who supported him at the time. Indeed, that subtle penetration into many sectors of the population through hygiene propaganda, together with the norms introduced by the 1888 health law, was undoubtedly responsible for the marked improvement in public health that took place in our country in the first decades of the 20th century. Sclavo always loudly proclaimed the right of hygiene to be regarded as a veritable branch of medicine and to be given the utmost consideration by those who guide the destiny of a country. He saw hygiene as preventive medicine, and was well aware that only through the work of an enlightened government and an educated population would it be possible to reach that public health threshold that is a fundamental requisite to solving many social problems* (Mazzetti 1949).

His teachings remain valid today, at the time of a pandemic of enormous proportions. Moreover, his life, his studies and the valuable projects that he brought to fruition led to the emergence of a new professional figure, the hygienist, at the turn of the century in a country afflicted by diseases due to infection and deficiency and in a public health context that can only be described as disastrous.

In his efforts to improve this situation, Sclavo strove to forge connections between medicine and the practical application of public health, thereby laying the foundations for preventive medicine.

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The Magnitude of Carbapenemase and ESBL Producing *Enterobacteriaceae* Isolates from Patients with Urinary Tract Infections at Tikur Anbessa Specialized Teaching Hospital, Addis Ababa, Ethiopia

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Abstract

Background The emergence of multidrug-resistant organisms, such as extended-spectrum beta-lactamase-producing *Enterobacteriaceae* (ESBL-PE), and carbapenemase-producing *Enterobacteriaceae* (CPE) is a public health concern. Therefore, this study aimed to determine the magnitude of carbapenemase and ESBL producing bacteria isolated from patients affected by Urinary Tract Infection (UTI).

Methods A cross-sectional study was conducted from December 2018 to March 2019 at Tikur Anbessa Specialized Hospital. A total of 120 *Enterobacteriaceae* isolates from UTI patients were collected and identified on species level using standard microbiological methods. Antimicrobial susceptibility test was determined according to the guidelines of the Clinical and Laboratory Standards Institute. Detection of ESBL production was carried out by using ESBL ChromoSelect Agar medium and the combined disk diffusion. Production of carbapenemase was determined by using Hodge-test and modified carbapenem inactivation method as described in CLSI guidelines.

Results Out of the total 120 *Enterobacteriaceae* isolates, 74 (61.7%) were ESBL-producers, and 8 (6.7%) were carbapenemase producers. The most common ESBL

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producing isolate was *E.coli* 38 (51.4%) and the most common carbapenemase-producing isolate was *K.pneumoniae* five (62.5%). Most of the ESBL and carbapenemase-producing isolates were recovered from hospitalized patients 46 (62.2%) and 7 (87.5%) respectively. The rate of ESBL and CPE production was observed high among patients taking antibiotics 64.8% (59/91) and 7.7% (7/91) respectively, but no significant association was observed $p > 0.05$. Furthermore, about 1.7% (2/120) isolates were found both ESBL and carbapenemase producers. Significant resistances rates were observed in ESBL and CPE isolates.

Conclusion *Enterobacteriaceae* isolates showed a significantly higher rate of ESBL production. A significant figure of carbapenemase production was observed among *Enterobacteriaceae* isolates causing UTI. The production of ESBL and CPE enhanced for an increased rate of MDR patterns. Efforts need to be made to introduce a system for tracking and detecting ESBL-PE and CPE-producing bacteria in hospitals, and monitoring dissemination of ESBL and CPE-producing *Enterobacteriaceae* is strongly recommended.

Keywords

Carbapenemase · *Enterobacteriaceae* · Extended-spectrum-beta-lactamase · Multidrug-resistant

1 Introduction

Enterobacteriaceae are common pathogens and causes of different types of community- and hospital-acquired infections (Rodríguez-Baño et al. 2018). *Enterobacteriaceae* easily acquire and transfer drug resistance genes through plasmids and transposons (Okoche et al. 2015). These families of bacteria are emerging as a public health threat in recent decades due to their production of extended-spectrum beta-lactamase (ESBL) and carbapenemase (Bharadwaj et al. 2018; Rodríguez-Baño et al. 2018; Behzadi et al. 2021).

Extended-spectrum beta-lactamases (ESBL) are enzymes produced by *Enterobacteriaceae* that are capable of conferring resistance to penicillins, first, second and third generationcephalosporins and aztreonam (but not cephamycins and carbapenems) and are inhibited by clavulanic acid, tazobactam and sulbactam. Genes encoding for ESBLs are located on mobile genetic elements (plasmids, integrons or transposons), but can also be found on the bacterial chromosome (Saliu et al. 2017; Flokas et al. 2017; Singh et al. 2016). This phenomena of emerging and expanding pattern of multiple drug resistance in ESBL producing bacteria even gets more challenging as most of pharmaceutical companies are increasingly turning away from participating in the development of new antibiotics (Gajdács 2019).

These ESBL-PE are associated with Urinary tract infections (UTIs) infecting an estimated 150 million people worldwide annually and UTIs are the third most common types of infections worldwide (Tamadonfar et al. 2019; Gajdács 2020). For the treatment of UTIs empiric therapies of cephalosporins (first, second, and third) generations, trimethoprim-sulfamethoxazole, amoxicillin-clavulanate, aminoglycosides, and piperacillin were recommended at different routes (Dayan et al. 2013).

However, the production of ESBL and CPE makes the treatment option narrow and genes encoding resistance to other antimicrobial agents, such as aminoglycosides and fluoroquinolones, are often found in proximity to the genes encoding ESBL on bacterial plasmids, thereby conferring multidrug resistance patterns (Dayan et al. 2013; Okoche et al. 2015).

Before the advent of CPE, carbapenems were the drug of choice for treating UTIs caused by multidrug-resistant *Enterobacteriaceae.* But the increasing resistance of *Enterobacteriaceae* to carbapenems has resulted in a lack of effective therapeutic alternatives for UTIs caused by carbapenem-resistant *Enterobacteriaceae* (Amladi et al. 2019). Mostly, resistance to carbapenems in *Enterobacteriaceae* is due to the production of carbapenemase enzyme that is capable of

hydrolyzing not only the carbapenems but also all the other beta-lactam antibiotics (Okoche et al. 2015).

The emergence of CPE resulted in the burden of MDR causing UTI mainly among *E. coli* and *K. pneumoniae*. These strains become a serious threat to public health, associated with high mortality rates and have the potential to spread widely. Infections are difficult and in some cases impossible to treat and have been associated with mortality rates of up to 50% (Eshetie et al. 2015; Gajdács et al. 2019).

In the study site data about the magnitude of ESBL and carbapenemase production among uropathogens is limited and well-organized data to follow the resistance pattern among *Enterobacteriaceae* is not sufficient. Therefore, this study aimed to determine the magnitude of carbapenemase-producing *Enterobacteriaceae* (CPE), ESBL producing *Enterobacteriaceae* (ESBL-PE) causing UTIs and current data on the local resistant patterns of ESBL-PE and CPE.

2 Materials and Methods

2.1 Data Collection

A hospital-based cross-sectional study was conducted from December 2018 to March 2019 at TASH. This hospital is one of the largest hospitals in Ethiopia located in the capital city of Ethiopia established in 1972, with over 800 beds and provides clinical services to 370,000–400,000 patients a year. The *Enterobacteriaceae* isolates used for this study were collected from patients with urinary tract infections using convenient sampling techniques.

2.2 Bacteria Isolation and Identification

Clean catch mid-stream urine samples of UTI suspected patients were inoculated into 5% sheep blood agar (BAP, Oxoid UK) and MacConkey agar plates. Inoculated agar plates were incubated aerobically at 35± 2 °C for 18 to 24 h. The cultured plates were examined for the growth and growth of pure colonies. Culture plates with mixed colonies were sub-cultured on 5% sheep blood agar and nutrient agar medium for the growth of isolated colonies. Bacterial identification was made by looking at the colony characteristics, gram staining, and by using standard microbiological methods (Vandepitte et al. 2003) S1 Table 1.

2.3 Antimicrobial Susceptibility Testing

The bacterial isolates were then tested for antimicrobial susceptibility *on Muller Hinton agar* (Oxoid UK) *using the Kirby–Bauer disc diffusion method following the* recommendation of the Clinical and Laboratory Standards Institute (CLSI 2018). The following antibiotics discs *from* BD USA company (*in μg/disk*) were used in the sensitivity test; ampicillin (10), gentamicin (10), amikacin (30), *tobramycin* (*30*), cefotaxime (30), ceftazidime (30), ceftriaxone (30), ciprofloxacin (5), trimethoprim/sulphamethoxazole (25), cefepime (30), cefoxitin (30), meropenem (10), Imipenem (10), amoxicillin/clavulanic acid (20/10), piperacillin-tazobactam (100/10) and cefuroxime (30). *After overnight incubation,* the results were interpreted as susceptible, intermediate or resistant according to CLSI guidelines (CLSI 2018). However, during the analysis of the data intermediate results were included with resistant one S2 Table 2.

2.4 Screening and Confirmation of ESBL

Detection of ESBL production was carried out by using ESBL ChromoSelect Agar medium (Sigma Aldrich, Germany) and the combined disk diffusion method. A 0.5 McFarland turbidity standard of a bacterial colony of overnight incubated pure culture was prepared and inoculated onto ESBL ChromoSelect agar base medium and Muller-Hinton agar for the combined disk diffusion method. The ESBL agar plates were incubated

Table 1 Socio-demographic and clinical characteristics of study participants

Variables	Category	Frequency	Percentage
Gender	Male	54	55.0
	Female	66	45.0
Age	Birth to <5 years	38	31.7
	5 years to <18 years	23	19.2
	18 years to <45 years	38	31.7
	≥ 45 years	21	17.5
Patient setting	Inpatient	75	62.5
	Outpatient	45	37.5
Ward type	Pediatric ward	25	20.8
	ICU	7	5.8
	Medical ward	33	27.5
	Outpatient wards	45	37.5
	Emergency	10	8.3
Organism identified	*Escherichia coli*	66	55.00
	Klebsiella oxytoca	9	7.50
	Klebsiella pneumoniae	37	30.83
	Others[a]	8	6.67

[a]*Enterobacter cloacae* (1), *Morganella morganii* (1), *Providencia stuartii* (2), *Proteus mirabilis* (3), and *Proteus vulgaris* (1). *ICU* intensive care unit

Table 2 Distribution of ESBL producing *Enterobacteriaceae* isolates among UTI patients in TASH, 2019

Variables		*E. coli*	*K. pneumoniae*	*K. oxytoca*	Others	Total *N*(%)	*P*-value (X^2)
Sex	Male	12	18	3	1	34(46)	0.792
	Female	26	9	3	2	40(54)	
Age	Birth to <5 years	12	11	2	0	25(33.8)	0.492
	5 to <18 years	2	6	2	1	11(14.8)	
	18 to <45 years	13	8	2	2	25(33.8)	
	≥ 45 years	11	2	0	0	13(17.6)	
Patient settings	In	21	18	4	3	46(62.2)	0.923
	Out	17	9	2	0	28(37.8)	
Ward type	Pediatrics	6	5	2	1	14(18.9)	0.943
	ICU	1	2	1	1	5(6.8)	
	Medical ward	13	7	0	1	21(28.4)	
	Emergency	1	4	1		6(8.1)	
Current antibiotics treatment	Yes	28	24	4	3	59(64.8)	0.519
	No	10	3	2	0	15(51.7)	
Exposure to antibiotics[a]	Carbapenems	2	2	1	0	5(71.4)	0.209
	Cephalosporin	16	3	14	1	34(59.6)	
	Fluoroquinolones	4	5	0	1	10(71.4)	
	Beta-lactamase inhibitor	5	2	0	1	8(80)	
Total (*n*,%)		38(51.4)	28(37.8)	6(8.1)	3(4.0)	74(100)	

Others = *Enterobacter cloacae* (1), *Providencia stuartii* (1) and *Proteus mirabilis* (1)
[a]In the past 3 months

aerobically at 37 °C for 18 to 24 h. Colonies of ESBL producers develop species-specific colors and interpreted them according to the manufacturer's instructions.

The combined disk diffusion method was performed by using cefotaxime (30 μg), and ceftazidime (30 μg) alone and then in combination with clavulanate (10 μg) on Muller-Hinton ager. After overnight incubation, a 5-mm increase in zone diameter for cefotaxime and ceftazidime tested in combination with clavulanate versus its zone when tested alone was considered indicative of ESBL production (CLSI 2018).

2.5 Detection of Carbapenemase Production

Isolates that were resistant and intermediate to either meropenem or imipenem were tested for production of carbapenemase by using the Hodge test (HT) and modified carbapenem inactivation method as described by (CLSI 2018; Rao et al. 2019). Briefly, HT was performed as follows, a 0.5 McFarland turbidity standard of ATCC *Escherichia coli* 25922 was prepared on nutrient broth (Oxoid, UK); lawn culture was made from the preparation onto the Mueller-Hinton agar plate. After drying a meropenem disc was placed at the center of the plate. The test strain and control strains were streaked at least 20-25 mm length from the edge of the meropenem disc to the periphery of the plate in different directions. The plates were incubated at 37 °C for 18–24 h. The presence of a cloverleaf type of zone of inhibition near the test organism was interpreted as HT positive (CLSI 2017; Rao et al. 2019).

Additionally, a modified carbapenem inactivation test was performed briefly, 1 μl loop full of test organism from overnight blood agar plate was suspended in 2 ml of nutrient broth and 10 μg of meropenem disk was added and incubated at 37 °C for 4 h. When the meropenem, test organism and nutrient broth incubation is about to end 0.5 mackferland suspension of *Escherichia coli* ATCC 25922 was prepared and inoculated on MHA. Meropenem disks from the nutrient broth was taken out and placed on MHA previously meropenem susceptible *Escherichia coli ATCC* 25922 strain inoculated and incubated overnight at 37 °C following incubation zone of inhibition was measured and interpreted as recommended by (CLSI 2018; Gajdács et al. 2020a, b).

2.6 Quality Control and Data Quality Assurance

The quality of microbiological methods used for bacterial identification was controlled by running *Klebsiella pneumoniae* ATCC® 700,603 and *Escherichia coli* ATCC® 25,922 strains for every new batch. *E. coli* ATCC® 25,922 and ATCC® 35,218 standard strains were used to check the quality of antibiotics. For the ESBL confirmatory test, *K. pneumoniae* ATCC® 700,603 (ESBLs positive) and *E. coli* ATCC® 25,922 (ESBLs negative) control strains were used to check the quality of used antibiotics disks. For carbapenemase confirmatory tests control strains *K. pneumoniae* ATCC® BAA-1705 (positive) and *E. coli* ATCC 25922 (negative) were used to control the effectiveness of the procedure.

2.7 Data Analysis

Before data entry data was checked, cleaned for any incomplete information, and double entered into Epidata software version 3.1, and then it was exported to SPSS version 25.0 software for analysis. *P*-value <0.05 was considered statistically significant.

2.8 Ethical Consideration

The study protocol, including consent procedure and ethical issues, was approved by Addis Ababa University, College of Health Sciences, Department of Microbiology, Immunology, and Parasitology Research Ethical Review committee (DRERC) (Ref. no. DERC/17/18/02-M) and Armauer Hansen Research Institute (AHRI/ALERT) Ethical Review Committee (AAERC)

(Ref.no P011/18). A support letter was obtained from the study site. Written informed consent was obtained from both adult study participants as well as from parents or guardians on the behalf of the children and newborn infants who participated in the study.

3 Results

3.1 Socio-Demographic Characteristics

A total of 120 study participants with UTI by *Enterobacteriaceae* were included in this study with the age range of 1 month to 90 years with the (mean + Standard Deviation) of (22 ± 1.9) years. The majority of the participants were female 66 (55%) and the sex ratio male to female was 1:1.2. About 75 (62.5%) of the participants were hospitalized patients. Furthermore, the majority of the participants were from age groups <5 years and 18 to 45 years 38 (31.7%) respectively; and 44% (33/75) and 33.3% (25/75) of the participants were from medical and pediatrics wards respectively Table 1.

3.2 The Magnitude of ESBL-Producing *Enterobacteriaceae*

Of the 120 uropathogens about 61.7% (74/120) were positive for ESBL. Additionally, from those ESBL producing isolates, *E.coli* (51.4%) and *K. pneumoniae* (36.5%) were the major organisms. The highest intra-species ESBL positivity was observed for *K. pneumoniae* (72.9%) followed by *K. oxytoca* (66.7%) and *E. coli* (57.6%). From the total ESBL positives, about 62.2% (46/74) was accounted by hospitalized patients. On the other hand, ESBL production from the community and hospitalized patients showed comparable magnitudes 62.2% (28/45) and 61.3% (46/75) respectively Table 2.

E. coli was the major ESBL producing *Enterobacteriaceae* isolates among UTI patients from outpatients and hospitalized patients 60.7% and 45.7% respectively. A higher rate of ESBL production was observed among patients who are on antibiotics 64.8% (59/91) than those who are not taking antibiotics 51.7% (15/29). Participants previously exposed to beta-lactamase inhibitor, carbapenems, and fluoroquinolones showed 80%, 71.4%, 74.1% ESBL positivity respectively than those who have not been taking any group of antibiotics (53.1%).

Generally, from the socio-demographic features studied no significant association was observed between ESBL production and the risk factors in Table 2.

3.3 The Magnitude of Carbapenemase-Producing *Enterobacteriaceae*

Out of 120 isolates of *Enterobacteriaceae* from UTI patients about 17 (14.2%) were suspected to be carbapenemase-producing *Enterobacteriaceae* (CPE): *K.pneumoniae* (64.7%, 11), *E.coli* (17.7%, 3), *K.oxytoca* (11.8%, 2) and one isolate of *P.mirabilis* (5.8%). These isolates were checked for the production of carbapenemase enzyme and about 47.1% (8/17) isolates were carbapenemase producers; thus, the overall magnitude CPE was 6.7% (8/120). From the eight carbapenemase-producing isolates, five (62.5%) were *K. pneumoniae,* and seven out of the eight (87.5%) CPE were isolated from hospitalized patients who have had taken antibiotics in the last 3 months and only one patient had no history of antibiotics use in the last 3 months. However, no significant association was observed between previous exposure to antibiotics and the production of carbapenemase ($P > 0.05$, at 95% CI). Out of 120 isolates of *Enterobacteriaceae,* two (1.7%) isolates exhibited both CPE and ESBL; and they were *K.pneumoniae* from hospitalized patients who have been previously exposed to cephalosporins (S3. Table 3).

3.4 Comparison of AST Patterns Between ESBL and CPE Producing and Non-producing Isolates

Enterobacteriaceae isolates with ESBL production were found more resistant to gentamicin (67.6% vs 50%, $P < 0.05$: 95% CI), trimethoprim/sulfamethoxazole (91.9% vs 86.9%), cefotaxime & ceftriaxone (98.7% vs 52.2%, $P < 0.05$: 95% CI), ceftazidime (94.6% vs 54.4%, $P < 0.05$: 95% CI) and ciprofloxacin (79.7% vs 58.7%, $P < 0.05$: 95% CI). Despites higher resistance of ESBL producing *Enterobacteriaceae* to the above antibiotics; ESBL producing isolates were found less resistant than to the non-producing isolates for antibiotics like amikacin (8.1 vs 17.4) %, meropenem (6.7 vs 19.6)%, imipenem (10.8 vs 19.6) %. Additionally, *Enterobacteriaceae* isolates with CPE production had higher resistance to aminoglycosides, cephalosporins, beta-lactam inhibitor combination, and quinolones (Table 3).

3.5 The Magnitude of Multidrug Resistance (MDR) Patterns

From the total isolates of *Enterobacteriaceae* about 109 (90.8%) were non-susceptible to ≥3 antimicrobials in different categories and therefore defined as MDR. Among the MDR isolates, about 7.3% (8/109) were resistant to all antibiotics tested; about 45.9% (50/109) isolates were resistant to six classes of antibiotics. Out of these 109 MDR isolates, about 52.3% and 33.9% of them were accounted by *E.coli* and *K.pneumoniae* respectively. In general, the highest rate of intraspecies MDR pattern was observed in *K.pneumoniae* 100% (37/37) and *K.oxytoca* 100% (9/9) followed by *E.coli* 86.4% (57/66). Additionally, the highest rate of MDR pattern was observed in patients previously exposed to carbapenem (100%), quinolones (92.8%), cephalosporins (91.2%), and penicillin groups (90%) than the non-exposed patients (87.5%).

Table 3 Comparison of AST patterns between ESBL and CPE producing and non-producing *Enterobacteriaceae* isolates at TASH

	ESBL-PE			CPE		
Antibiotics	Positive	Negative	*P*-value	Positive	Negative	*P*-value
Ampicillin	100	93.5	**0.02**	100	97.3	0.6
Gentamicin	67.6	50	**0.05**	50	50	0.5
Amikacin	8.1	17.4	0.12	25	10.7	0.2
Cefotaxime	98.7	52.2	**0.05**	100	79.5	0.2
Ceftriaxone	98.7	52.2	**0.05**	100	79.5	0.2
Ceftazidime	94.6	54.4	**0.05**	100	77.7	0.1
Ciprofloxacillin	79.7	58.7	**0.01**	87.5	70.5	0.3
Tobramycin	55.4	54.4	0.9	62.5	54.5	0.6
Cefipeme	94.6	54.4	**0.05**	100	77.7	0.1
Cefoxitin	66.2	73.9	0.4	100	67	**0.05**
Imipenem	10.8	19.6	0.2	100	8	**0.05**
Meropenem	6.8	19.6	0.12	100	5.4	**0.05**
Trimethoprim/sulphamethoxazol	91.9	86.9	0.4	100	89.3	0.3
Pipracillin-tazobactam	56.5	51.4	0.5	87.5	50	0.04
Amoxacillin/clavulanic acid	91.9	89.1	0.6	100	90.2	0.3
Cefuroxime	100	76.1	**0.05**	100	90.2	0.3

ESBL-PE extended spectrum beta-lactamase producing Enterobacteriaceae, *CPE* carbapenemase producing *Enterobacteriaceae*

3.6 Comparison of MDR Pattern Between ESBL and CPE Producing and Non-producing *Enterobacteriaceae*

Out of 74 ESBL producing *Enterobacteriaceae* isolates, 100% were found to be MDR which was much higher than none producing isolates about 35/46 (76.1%). When we compare the magnitude of MDR patterns among ESBL producing *Enterobacteriaceae* species; ESBL-*E.coli* isolates were found to be 100% (38/38) MDR as compared to the non-producers 19/28 (67.8%). However, both ESBL producing and none producing isolates of *K.pneumoniae* and *K.oxytoca* were found to be 100% MDR as shown in Table 4. Additionally, isolates with carbapenemase production were found 100% MDR than the non-producers about 90.2(101/112).

4 Discussion

In our study, the overall magnitude of ESBL production was (61.7%) which was higher than studies conducted in Ethiopia, Adama (25%) (Selassie 2016), Addis Ababa (50.7%) (Teklu et al. 2019), and other parts of the world such as in France 4% (De La Blanchardière et al. 2015), in Jordan 46%(Albaramki et al. 2019). Among the possible causes for this high magnitude of ESBL production could be due to uncontrolled use and prophylaxis of UTIs by cephalosporin (Dayan et al. 2013), variation in the application of proper infection prevention measures and the variation in the season at which the study was conducted. Additionally in some of *Enterobacteriaceae* intrinsic resistance patterns also plays an important role as described by (Gajdács et al. 2020a, b). A magnitude of 61.7% of ESBL production was comparable with a 58% report in Burkina Faso (Ouedraogo et al. 2016).

In our, study *E.coli* was a major ESBL producer (51.4%) than *K.pneumoniae* (36.5%) as a comparable figure was reported in Jimma Ethiopia (Abayneh et al. 2018). Patients exposed to antibiotics in the past 3 months showed the highest rate of ESBL production similar finding was reported in French (Flateau et al. 2018), Amsterdam (Reuland et al. 2016), and Japan

Table 4 Comparison of AST and MDR patterns between ESBL producing and none producing *Enterobacteriaceae* isolates in TASH

	Rate of resistance pattern between ESBL & non-ESBL isolates							
	E.coli		*K.pneumoniae*		*K.oxytoca*		Others	
Antibiotics	ESBL$^+$	ESBL$^-$	ESBL$^+$	ESBL$^-$	ESBL$^+$	ESBL$^-$	ESBL$^+$	ESBL$^-$
Ampicillin	100	89.3	100	100	100	100	100	100
Gentamycin	60.5	35.7	70.3	90	83.3	66.7	100	40
Amikacin	7.9	10.7	3.7	20	16.7	66.7	33.3	20
Cefotaxime	97.4	35.7	100	100	100	66.7	100	40
Ceftriaxone	97.4	35.7	100	100	100	66.7	100	40
Ceftazidime	94.7	35.7	96.3	100	83.3	66.7	100	60
Ciprofloxacin	86.8	46.4	66.7	100	83.3	100	100	20
Tobramycin	50	39.3	51.8	80	83.3	100	100	60
Cefipeme	97.4	39.3	96.3	100	100	66.7	66.7	40
Cefoxitin	68.4	60.7	59.3	100	66.7	100	100	80
Imipenem	2.6	7.1	22.2	50	16.7	33.3	0	20
Meropenem	0	7.1	14.8	50	16.7	33.3	0	20
Trimethoprim-sulphamethoxazol	86.8	85.7	96.3	100	100	100	10	60
Pipracillin-tazobactam	47.3	42.8	59.3	100	50	66.7	0	40
Amoxicillin-clavulanic acid	94.7	85.7	85.2	100	100	100	100	80
Cefuroxime	100	64.3	100	100	100	66.7	100	100
MDR pattern	100	67.8	100	100	100	100	100	40

Keys: *ESBL*$^+$ ESBL positive, *ESBL*$^-$ ESBL negative
Others = *E.cloacae* (1), *P.stuartii* (1), *P.mirabilis* (1)

(Nakai et al. 2016). Even if, there was no significant association between exposure to antibiotics and production of ESBL in our study, the finding could be a supportive argument for those who say previous antibiotics treatment is a risk factor for colonization by ESBL producing *Enterobacteriaceae*.

Among 120 *Enterobacteriaceae* isolates, 8 (6.7%) were found to be carbapenemase producers which were higher than a study conducted in the UK (Woodford et al. 2018), Ethiopia (Eshetie et al. 2015). However, our finding is lower than other reports from India (Rao et al. 2019) in Uganda (Okoche et al. 2015), and Ethiopia (Legese et al. 2017). This variation could be due to previous exposure of patients to broad-spectrum antibiotics (Eshetie et al. 2015), and the nature of patients included in our study, study setting, and methodology applied for detection of carbapenemase enzymes.

In our study, *K.pneumoniae* isolates were the most common carbapenemase-producing species. This result is in agreement with other studies (Rao et al. 2019; Okoche et al. 2015) that magnify the role of carbapenemase-producing *K. pneumoniae* isolates in the rise of antibiotics resistance rates. To this end carbapenemase-producing *K. pneumoniae* are emerging global threats because in most case carbapenemase genes are associated with mobile genetic elements that can spread horizontally within and between bacterial species and *K. pneumonia* has been identified as a crucial entry point of antibiotic resistance genes into the *Enterobacteriaceae* (David et al. 2019).

ESBL production has enhanced for co-resistance to different classes of antibiotics, gentamicin (67.6% vs 50%), ciprofloxacin (79.7% vs 58.7%), trimethoprim/sulphamethoxazole (91.9% vs 86.9%), ampicillin (100% vs 93.4%), cefepime (94.6% vs 54.4%) and cefuroxime (100% vs 76.1%) than none producers. This high resistance of ESBL producing *Enterobacteriaceae* to the above antibiotics was in close agreement with other studies done in Ethiopia (Teklu et al. 2019), Burkina Faso (Ouedraogo et al. 2016), in Saudi Arabia (Hassan and Abdalhamid 2014). This may be because ESBLs are often encoded by genes located on plasmids, that may carry genes for resistance to other antimicrobial agents such as aminoglycosides, trimethoprim/sulphamethoxazole, and fluoroquinolones (Fernando et al. 2017).

However, ESBL producers were least resistant to amikacin (8.1% vs 17.4%), meropenem (6.8% vs 19.6%), and imipenem (10.8% vs 19.6%) than the non-producer which was in close agreement with studies done in Ethiopia to amikacin (Abayneh et al. 2018) and amikacin and meropenem (Teklu et al. 2019). This is because amikacin was not a treatment of choice for infections caused by *Enterobacteriaceae* and the carbapenems were expensive and less commonly used in the Ethiopian context.

In the present study, the overall magnitude of MDR was 90.8%, higher than reports from other studies conducted in Ethiopia, Addis Ababa (68.3%) (Teklu et al. 2019), and Jimma Ethiopia (30.2%) (Gashaw et al. 2018). Several factors could account for this, including the unique nature of patients visiting TASH after exoposure to different antibiotics, 90.8% was fairly comparable with a study done in Ethiopia 87.4% (Eshetie et al. 2015) and Iraq 86.9% (Pishtiwan and Khadija 2019).

The present study showed that *Klebsiella spp* (100%) and *E.coli* (86.4%) were MDR among the *Enterobacteriaceae* isolates which was similar to studies done in Ethiopia (Eshetie et al. 2015), Nepal (Chander and Shrestha 2013), and Iraq (Pishtiwan and Khadija 2019). The magnitude of MDR in ESBL producing isolates was much higher than none producers (100% vs 76.1%). Similarly, a higher rate was reported in Thailand (Kiddee et al. 2019). ESBL production is significantly associated with MDR patterns ($p < 0.05$). And also those carbapenemase-producing *Enterobacteriaceae* were found 100% MDR than the non-producers (90.2%). However, ESBL producing and non-producing *Klebsiella* isolates showed similar MDR patterns.

5 Conclusion and Recommendation

The magnitude of ESBL producing *Enterobacteriaceae* isolates from UTI patients was found

high. The magnitude of carbapenemase production was observed rising in the study site among UTI patients. Additionally, an MDR pattern was observed in most of the isolates in this study particularly isolates with ESBL and CPE. Amikacin and meropenem had shown a good *in vitro* activity to those ESBL producing and non-producing groups of *Enterobacteriaceae*. For those *Enterobacteriaceae* producing carbapenemase, amikacin was found the most active antibiotics. Therefore, routine screening for ESBL and carbapenemase production is strongly recommended for better diagnosis of patients. Tracking of ESBL and carbapenemase-producing *Enterobacteriaceae* is recommended to contain the spread of those 'superbugs' in the community.

Limitation The followings are the limitation of this study

- We are unable to determine the level of ESBL, AmpC and CPE co-expression due to the unavailability of confirmatory tests for AmpC
- Even if those methods used for detection of ESBL and carbapenemase are recommended by CLSI 2018 still they have their limitation as most phenotypic methods.

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Dataset The datasets supporting the conclusions of this article are included within the article and its additional files

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Correction to: Ultrastructural and Immunohistochemical Diagnosis of a Neonatal Herpes Simplex Virus Infection Presenting as Fulminant Hepatitis: A Case Report

Valentina Papa, Nunzio Cosimo Mario Salfi, Roberta Costa, Ilaria Bettocchi, Emilia Ricci, Duccio Maria Cordelli, Francesca Locatelli, Fabio Caramelli, and Giovanna Cenacchi

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This chapter was inadvertently published with the co-author name incorrectly spelt as Nunzio Cosimo Maria Salfi instead of it being rightly spelt as Nunzio Cosimo Mario Salfi. This has now been corrected.

The updated version of this chapter can be found at https://doi.org/10.1007/5584_2021_659

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Correction to: Achille Sclavo (1861–1930) and His Innovative Contributions to Italian Preventive Medicine and Healthcare Policy

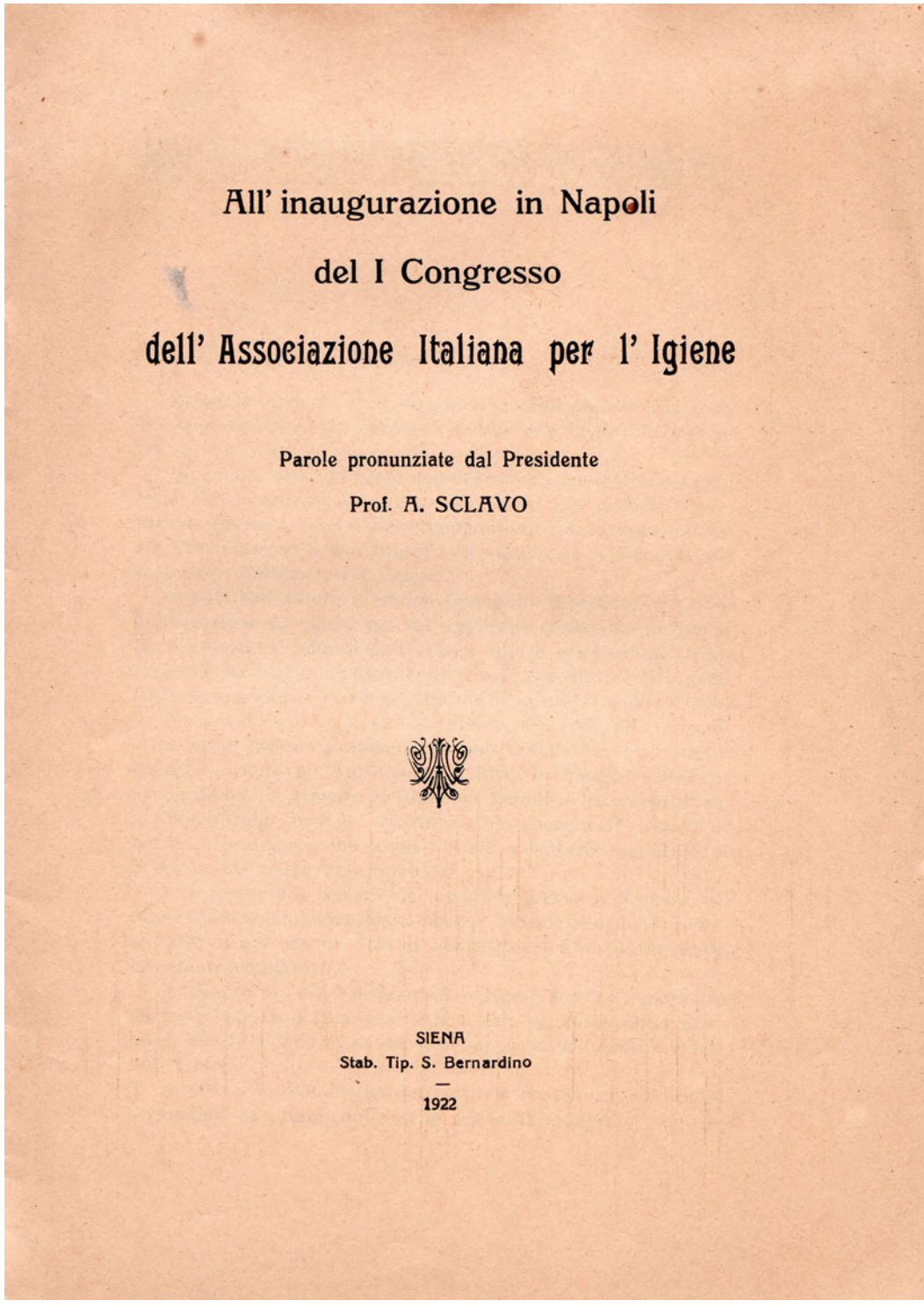
Mariano Martini and Davide Orsini

Correction to: Chapter "Achille Sclavo (1861–1930) and His Innovative Contributions to Italian Preventive Medicine and Healthcare Policy" in: M. Martini et al., Adv Exp Med Biol - Advances in Microbiology, Infectious Diseases and Public Health https://doi.org/10.1007/5584_2021_673

This chapter was inadvertently published without including the missing figures. This has now been corrected and the missing 3 figures have been inserted in their correct positions. The missing figures which has now been included are as follows:

Fig. 1 Achille Sclavo (Elderly Group Archive, Sclavo, Siena)

The updated version of this chapter can be found at https://doi.org/10.1007/5584_2021_673


All' inaugurazione in Napoli

del I Congresso

dell' Associazione Italiana per l' Igiene

Parole pronunziate dal Presidente

Prof. A. SCLAVO

SIENA

Stab. Tip. S. Bernardino

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1922

Fig. 2 Speech of 1921 by Achille Sclavo at the 1st Congress of the Italian Association for Hygiene (Elderly Group Archive, Sclavo, Siena)

Fig. 3 Printed advertisements Jodogelatina Sclavo, 1914 (Elderly Group Archive, Sclavo, Siena)

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