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Research Article **Araştırma Makalesi**

A New Strategy: Antibiotic Circumstances for Rational Drug use Against Clinical Aeromonas hydrophila And Bacterial Properties of This Bacteria on Different Agars

Yeni Bir Strateji: Klinik *Aeromonas hydrophila*'ya Karşı Akılcı İlaç Kullanımı İçin Antibiyotik Durumları ve Bu Bakterilerin Farklı Agarlar Üzerindeki Bakteriyel Özellikleri

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Abstract: Antibiotic resistance of *A. hydrophila* was once again observed on different agars. Random antibiotics drug treatment of diseases causes development resistance. Thus, we have faced post-antibiotic era in which our ability to challenge bacteria has diminished and the need for new strategies to deal with disease has increased. *A. hydrophila* ATCC reference strain, which causes the fatal Motil Aeromonas Septicemia (MAS) Disease in fish, was used in the study Colony structure of *A. hydrophila* formed on MacConkey (MAC), Aeromonas Isolation Base Agar (AIBA), Congo Red Agar (CRA) and Blood Agar (BA) were examined. The antibiotic susceptibility was determined by using the Kirby-Bauer method Mueller–Hinton Agar, MAC, AIBA and CRA agar plates. *A. hydrophila* was found sensitive to ciprofloxacin, enrofloxacin, gentamicin, sulphamethoxazole/trimethoprim, and resistant to penicillin G and oxacillin. The important difference was obtained as resistant to enrofloxacin on MAC. Multiple antibiotic resistance index (MARI) of *A. hydrophila* was determined as 0.33 in MHA and 0.5 in MAC. This difference was due to the antibiotic enroflaxin, and its appearance in this study, where a different new approach was tried for the first time, also added originality to the subject. This status may be related to an acquired plasmid. It is important to try to see resistance of *A. hydrophila* by using different agars while innovations such as the AntibiogramJ program are being tried to be added to antibiotic literature.

Keywords
- Motil aeromonas septicemia (MAS) disease
- Antibiotic resistance
- Aeromonas Isolation Base Agar (AIBA)
- Congo Red Agar (CRA)

Özet: Rastgele antibiyotiklerle hastalıkların ilaçla tedavisi direnç gelişimine neden olmaktadır. Böylece bakterilerle mücadele yeteneğimizin azaldığı ve hastalıklarla mücadelede yeni stratejilere olan ihtiyacın arttığı antibiyotik sonrası dönemle karşı karşıya kalmış durumdayız. Araştırma makalesinde, balıklarda ölümcül Motil Aeromonas Septicemia (MAS) Hastalığına neden olan, *A.*

Anahtar kelimeler
- Motil aeromonas septisemi (MAS) hastalığı
- Antibiyotik direnci

hydrophila ATCC referans suşu kullanılmıştır. MacConkey (MAC), Aeromonas Isolation Base Agar (AIBA), Congo Red Agar (CRA) ve Blood Agar (BA) üzerinde oluşan *A. hydrophila* kolonileri incelenmiştir. Antibiyotik duyarlılığı Kirby-Bauer yöntemi Mueller-Hinton Agar, MAC, AIBA ve CRA agar plakları kullanılarak belirlendi. *A. hydrophila* siprofloksasin, enrofloksasin, gentamisin, sülfametoksazol/trimetoprim'e duyarlı bulunurkan penisilin G ve oksasiline dirençli bulunmuştur. Önemli bir fark olarak *A. hydrophila*'nın MAC'ta enrofloksasine dirençli olduğu görülmüştür. *A. hydrophila*'nın çoklu antibiyotik direnç indeksi (MARI) MHA'da 0,33, MAC'da 0,5 olarak belirlendi. Bu farklılık enrofloksasin antibiyotiğinde tespit edilmiş ve ilk kez farklı bir yaklaşımın denendiği bu çalışmada bu durumun ortaya çıkması konuya özgünlük de katmıştır. Bu durumun edinilmiş bir plazmid ile ilgili olabileceği kanaatindeyiz. AntibiogramJ programı gibi yenilikler antibiyotik literatürüne kazandırılmaya çalışılırken, farklı agarlar kullanılarak A. hydrophila'nın direncinin görülmeye çalışılması önemlidir. *A. hydrophila*'nın antibiyotik direnci farklı agarlarda bir kez daha gözlendi.

- Aeromonas Isolation Base Agar (AIBA)
- Congo Red Agar (CRA)

1. INTRODUCTION

Aeromonas hydrophila bacteria are Gram-, facultative, anaerobic straight rods with rounded ends (Sawyer, 2020*). A. hydrophila*, with its cytotoxic and hemolytic exoenzymes, causes infections such as gastroenteritis, vomiting, fever and epigastric pain, as well as septicemia, arthritis, meningitis and peritonitis in humans. *A. hydrophila* complex cause a hemorrhagic septicemia in fish (Fernández-Bravo and Figueras, 2020). Therefore, it has been recently described as "jack-of-all-trades" (Rasmussen-Ivey et al., 2016) alive creatures comorbidity (Janda and Abbott, 2010). This bacteria expressed this way because it is a powerful pathogen in terms of virulence.

Motile Aeromonas Septicemia (MAS) Disease is caused by *A. hydrophila* agent. Gross pathology of MAS can range from few external or internal symptoms in peracute cases to hemorrhagic septicemia, in acute cases, to abscesses, serious wounds, fibrinous peritonitis (Kousar et al., 2022) and ulcers in chronic cases. Severe MAS outbreaks frequently display a range of lesions indicating variation in progression of ailment in fish. Ailment symptoms include septicemia, reddened fins, anus inflammation, tissue hemorrhages, exophthalmia and dropsy in fish. Oedema of scale pockets, scale loss, fin erosion and assist (dropsy) are visible (Hanson et al., 2019; Liu et al., 2020; Mahboub and Tartor, 2020). The agent can survive in mud for 2 months. There is no specific season in which the disease occurs. Morbidity can reach 100%. However, the disease is not absolutely fatal. Mortality does not exceed 40 - 50% even in very adverse environmental conditions for fish. Oxytetracycline 50-75 mg/kg (c.a.) for treatment purposes. It is recommended orally for 10 days. Treatment in line with the antibiogram results; Sulfamerazine at a dose of 200-300 mg / kg (c.a.) for 10 days, Chloramphenicol at 50-70 mg / kg. It can be used at (c.a.) dosage for 5 – 10 days (Mahboub and Tartor, 2020).

The present study aimed to test growth characteristics of *A. hydrophila* on different agar and antibiotic susceptibility in media. Also i t was aimed to test the colony morphology characteristics of *A. hydrophila* on different agar, antibiotics resistance and post-antibiotic epoch antibiotic susceptibility, in media. Additionally aim of the study was to test antibiotic sensitivity , colony morphology differences, biofilm formation of *A. hydrophila* causing MAS was tested on different agars, firstly Mueller-Hinton Agar (MHA) known as Kirby-Bauer Disk Diffusion assay (Antibiogram assay). For this purpose antibiotic susceptibility of *A. hydrophila* was tested on MacConkey (MAC), Aeromonas Isolation Base Agar (AIBA) and Congo Red Agar (CRA) agars. Furthermore , we determined multi-antibiotic resistance of A. hydrophila and investigated the biofilm of this pathogen in

CRA. Also we investigated the colony differences by monitoring the colony morphology of *A. hydrophila* on MAC, AIBA, CRA and Blood Agar (BL) agars.

Briefly, the aim of this study is to emphasize once again the antibiotic sensitivity with the Kirby-Bauer Disk Diffusion test (Antibiogram assay) using Mueller-Hinton Agar (MHA), which is the well-established antibiotic test in the literature as an invariable rule. *A. hydrophila* bacteria was also grown on other agars to determine the antibiotic profile in other agars as well. A dditionally, the present study aimed to introduce practices that give more robust results, such as AntibiogramJ studies on the digital platform, instead of normal ruler measurements, accordingly AntibiogramJ study was carried out as a continuation of this study. Biofilm virulence is an element that remains in chronic diseases, implants, scales, inanimate tissues, and the environment to which it adheres even when the bacteria is not present any longer . Therefore, this study, focused on the profiles of *A. hydrophila* on different agars, and its virulence on CRA was investigated.

2. MATERIALS AND METHODS

2.1. Start-up

A. hydrophila reference strain was used throughout this study. *A. hydrophila* was grown in BHI maintained as frozen stock at -20°C (Stecchini et al., 1993). *A. hydrophila* was studied by bacteriological tests. The strains were identified morphologically and biochemically. In Gram staining assay, coc-shaped Gram+ bacteria Staphylococcus warneri was used as a control strain.2.2. Gram Staining

Bacteria cell strains wer e Gram stained according to proc edure (Bruckner, 2021).

2.3. Inoculum

A. hydrophila was grown on Tryptic Soy Agar (TSA), MacConkey (MAC), Aeromonas Isolation Base Agar (AIBA), Congo Red Agar (CRA) and Blood Agar (BA). Inoculate all plates (TSA, MAC, AIBA, CRA, BA) with test organisms and incubate at 25^{o}C for 24 h aerobically.

2.4. Biofilm on Congo Red Agar (CRA)

CRA medium was prepared with brain heart infusion broth 37 g/L, sucrose 50 g/L, agar 10 g/L and Congo Red indicator 0,8 g/L. Then it was added to the autoclaved BHAI with sucrose. Plates were then observed for dry crystalline black colonies for biofilm producers and red colonies indicating non-biofilm producers (Freeman et al., 1989).

2.5. Antibiogram Assay

A. hydrophila was tested for antibiotic resistance by using Kirby-Bauer method according to the Clinical Laboratory Standards Institute (CLSI, 2016) guidelines (Clinical L.S. Institute, 2016; Mueller and Hinton, 1941). Briefly, Muller-Hinton agar and a panel of 6 antibiotics disks were selected for resistance tests. These 6 antibiotic discs (Oxoid) were ciprofloxacin CIP (5 μg), enrofloxacin (ENR) (5 μg), gentamicin (CN10) (10 μg), penicillin G (P10) (10 μg), sulphamethoxazole/trimethoprim (SXT25) (25 μg), Oxacillin (OX1) (1 μg).

2.6. Multi Antibiotic Resistance Index (MARI)

This was carried out as described with a slight modification. MARI = resistant antibiotics ⏐ total antibiotics tested. MARI values> 0.2 indicate existence of isolate(s) from high – risk contaminated source with frequent use of antibiotics while valuesδ 0.2 show bacteria from source with less antibiotics usage (Nguyen et al., 2024).

3. RESULT

3.1. Start-up

The phenotypic characteristics of the *A. hydrophila* strain were determined by conventional assays (Table 1).

Table 1. Phenotypic characteristics of *A. hydrophila* strain.

Assay	Result
Gram stain	-
Morphology	Basil
Motility	+
Catalase	+
Oxidation/Fermentation(O/F)	+/+
Sensitivity to O/129	Resistance (R)

3.2. Gram Staining

S. warneri strain, a $Gram^+$ bacteria, was used as a negative control. *A. hydrophila* were $Gram^-$, whereas *S. warneri* were $Gram^+$ cocci ($^-$control) (Figure 1).

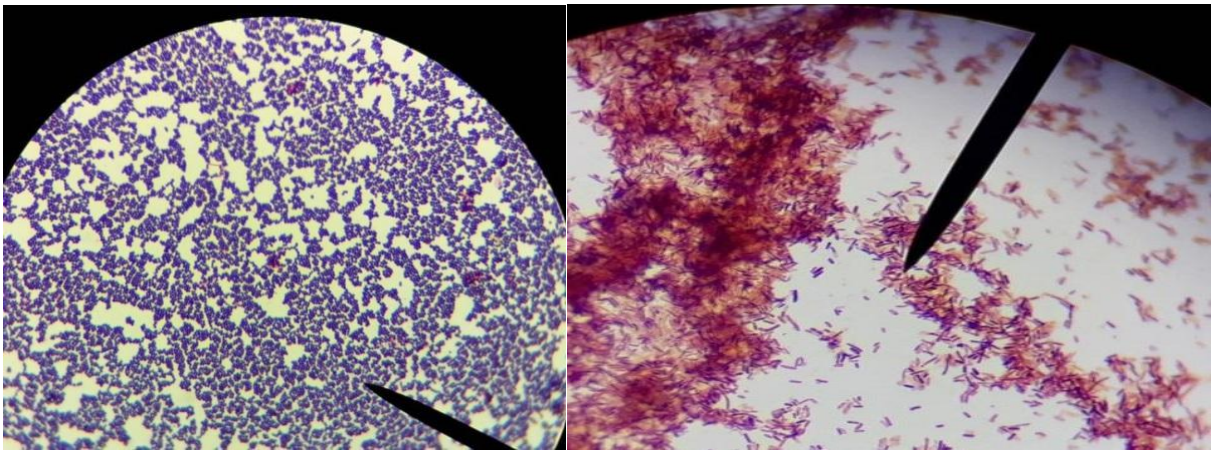
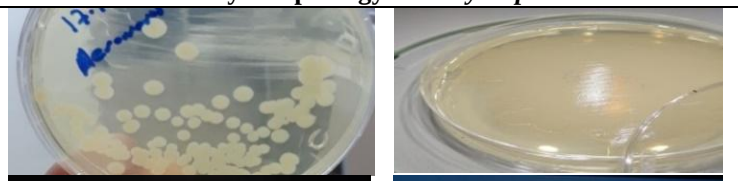
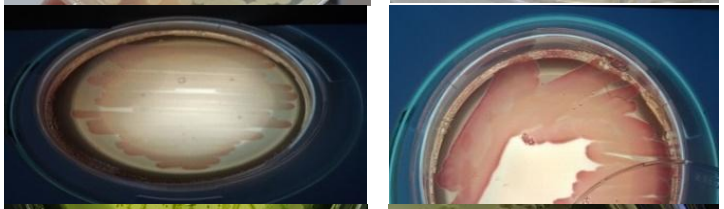
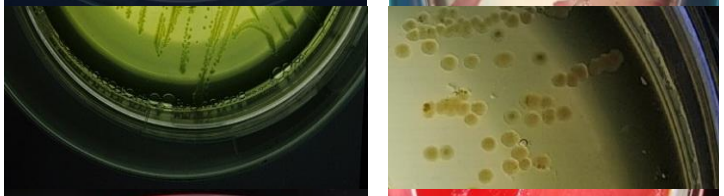


Figure 1. Gram staining of *S. warneri* ($^-$control). Gram staining of *A. Hydrophila*.

3.3. Inoculum After

A. hydrophila strain produced cream color colonies on TSA. Lactose posture of *A. hydrophila* monitored in MAC agar. *A. hydrophila* strain produced dark green colonies on AIBA. *A. hydrophila* strain produced red and occasionally weak black colonies on CRA. In BA, *A. hydrophila* that are 1-3 mm in diameter grayish color due to β-hemolysis and dark cream. In BA, colonies are β-hemolytic (Table 2).

Table 2. Colony structures of *A. hydrophila* on TSA, MAC, AIAB, CRA, BL agars.

Bacteria	Medium	Temperature- time	Colony structures	Colony morphology of *A. hydrophila*
A. hydrophila	TSA	25°C – 24 h	Growth: Luxuriant Color of colonies: Cream Colony structure: Big, characteristic colony	
A. hydrophila	MAC	25°C – 24 h	Growth: Luxuriant Color of colonies: Colorless pink and occasionally dark pink Colony structure: Big, characteristic colony	
A. hydrophila	AIBA	25°C – 24 h	Growth: Luxuriant Color of colonies: Dark green Colony structure: Opaque with dark centre, characteristic colony	
A. hydrophila	CRA	25°C – 24 h	Growth: Luxuriant Color of colonies: Red, black and occasionally weak black colonies Colony structure: Opaque, characteristic colony	
A. hydrophila	BA	25°C – 24 h	Growth: Luxuriant Color of colonies: cream and occasionally greenish gray Colony structure: Big, circular and convex, β-hemolysis colony	

3.4. Biofilm on Congo Red Agar (CRA)

In this study, in which colony morphology of *A. hydrophila* bacteria on different agars was also evaluated, biofilm was determined in CRA assay, which shows biofilm on agar rapidly in 24 hours. It was determined that *A. hydrophila* bacteria formed a black pigmented colony on the CRA and formed a biofilm (Figure 2).

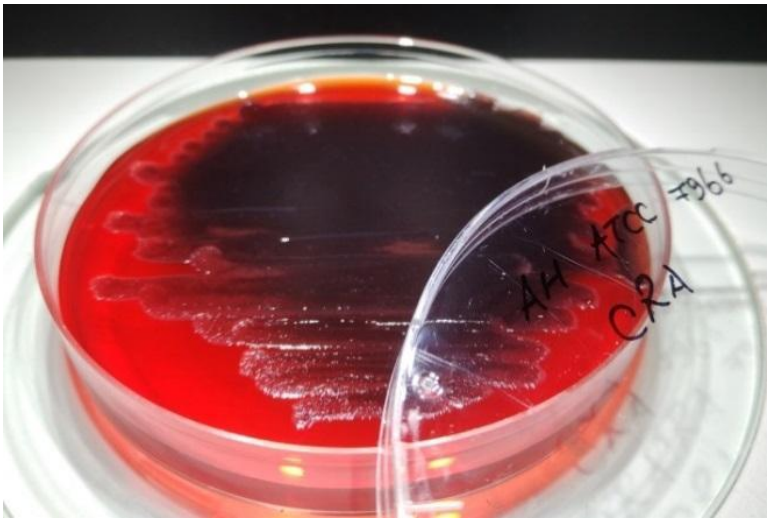


Figure 2. Biofilm production of *A. hydrophila* on CRA.

3.5. Antibiogram assay results

A. hydrophila was sensitive to ciprofloxacin, enrofloxacin, gentamicin sulphamethoxazole/trimethoprim, and was resistant to penicillin G and oxacillin (Figure 3). The resistance of *A. hydrophila* to 2 tested antimicrobial agents is shown in Table 3.

Figure 3. Sensitivity test of *A. hydrophila* to antibiotics on MHA.

3.6. Antibiogram Assay on MAC, AIBA and CRA Agars

Antibiotic sensitivity on MHA is an invariable fact in the literature. For this reason, the antibiotic susceptibility examined in other agars are presented separately in the article, as they are alternatives.

A. hydrophila was sensitive to ciprofloxacin, enrofloxacin, gentamicin sulphamethoxazole/trimethoprim, and was resistant to penicillin G and oxacillin (Table 3). However, *A. hydrophila* was resistant to penicillin G, enrofloxacin and oxacillin on MAC agar (Fig. 4). The sensitivity and resistance of *A. hydrophila* to antibiotics were shown on MAC (Fig. 4), AIBA (Fig. 5) and CRA agars (Fig. 6).

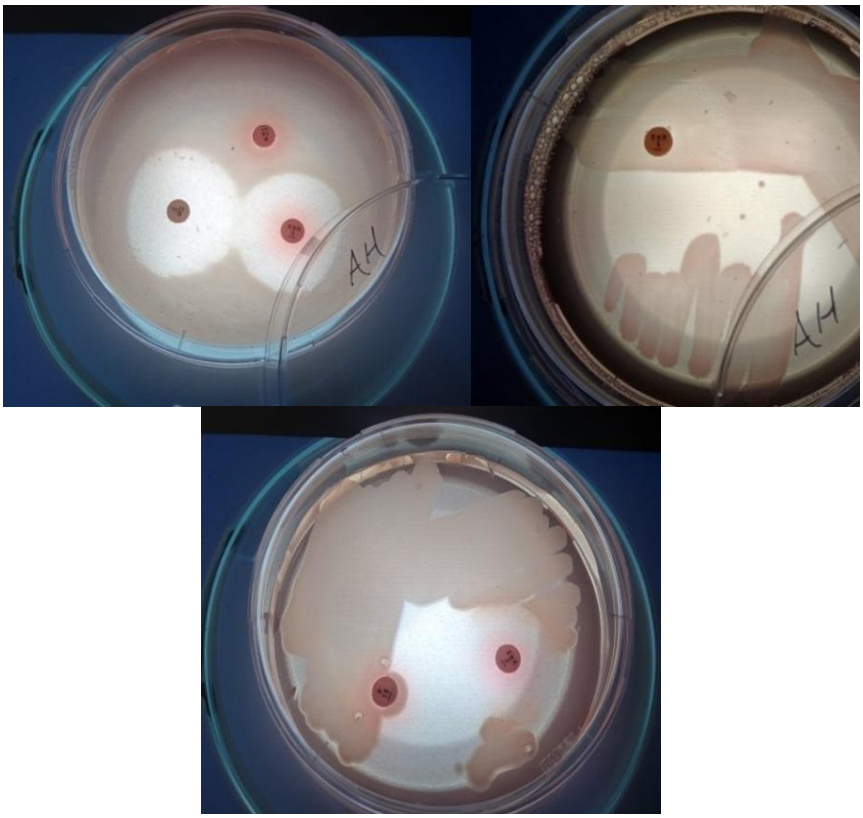


Figure 4. Sensitivity test of *A. hydrophila* to antibiotics on MAC.

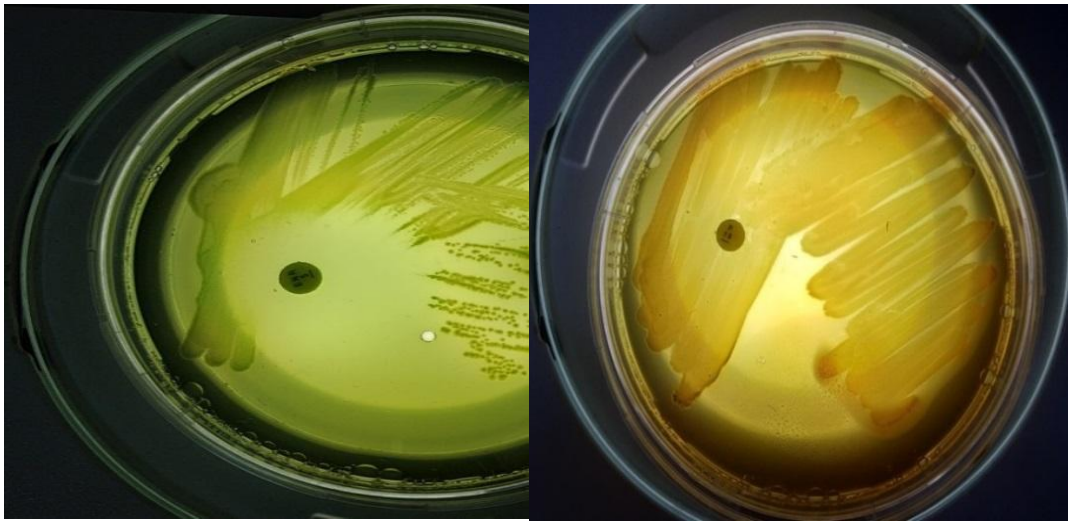


Figure 5. Sensitivity test of *A. hydrophila* to antibiotics on AIBA.

Figure 6. Sensitivity test of *A. hydrophila* to antibiotics on CRA.

Table 3. The antibiotic sensitivity and resistance of *A. Hydrophila*.

Antimicrobial Agent	Code	Disc Content	Test Organisms	Zone Diameter Posture Resistance (R) Intermediate (I) Sensitive (S)			
				MHA	MAC	AIBA	CRA
Ciprofloxacin	CIP5	5 µg	*A. hydrophila*	S (35)	S (35)	S (35)	S (35)
Gentamicin	CN10	10 µg	*A. hydrophila*	S (20)	S (20)	S (20)	S (20)
Enrofloxacin	ENR5	5 µg	*A. hydrophila*	S (30)	S (30) / R (0)*	S (30)	S (30)
Penicillin G	P10	10 µg	*A. hydrophila*	R (0)	R (0)	R (0)	R (0)
Sulphamethoxazole/Trimethoprim	SXT25	25 µg	*A. hydrophila*	S (25)	S (25)	S (25)	S (25)
Oxacillin	OX1	1 µg	*A. hydrophila*	R (0)	R (0)	R (0)	R (0)

*Very important difference

3.7. Multi Antibiotic Resistance index (MARI)

MARI = resistant antibiotics ⏐total antibiotics tested. MARI was 0.33 for MHA. MARI was 0.5 for MAC (Table 4).

Table 4. Multi Antibiotic Resistance index of *A. Hydrophila*.

Test organisms	Multi Antibiotic Resistance index (MARI)			
	MHA	MAC	AIBA	CRA
A. hydrophila	0.33	0.5	0.33	0.33

4. DISCUSSION

There are many agents, methods, causes used for fighting against diseases that have entered the literature. Antibiotics are the most effective elements in treatment of diseases. However, the uncontrolled use of antibiotics has resulted in antibiotic resistance and this is an important problem (Woo *et al*., 2022).

The formation of a community on the surface to which bacteria attach and become mass covered by extracellular polymers is called biofilm. Biofilm is a complex polymicrobial community that can contain different types of bacteria, is surrounded by a polysaccharide matrix produced by these bacteria, and can adhere to surfaces. There are numerous assays used to detect and quantify biofilms in bacteria (Moori *et al*., 2019; Rabha *et al*., 2021). Biofilm is virulence managed by an interbacterial communication system called Quorum Sensing System (QS) (Nurcan, 2010). Black colony was found in *Aeromonas sobria* on CRA (Filik, 2019). Abdulaal in 2019 reported that *A. hydrophila* produced biofilm on CRA. Likewise, in this study, *A. hydrophila* formed a biofilm on CRA.

Antibiogram testing is performed on MHA agar, which is known to be the unchangeable and unalterable rule. AntibiogramJ is the most complete software tool for antibiogram analysis without requiring any particular hardware system. Thanks to features of AntibiogramJ, researchers easily detect when automatic reading has failed and fix it to obtain correct results (Alonso et al., 2017). Here we like to emphasize antibiotic resistance on other agars, similarly to AntibiogramJ approach.

MacConkey agar (MAC) is a bacterial culture medium named after bacteriologist Alfred T. MAC (1861-1931). MAC is selective and differentiating agar that only grows Gram$^-$ bacteria it can further differentiate Gram$^-$ based on their lactose metabolism (Elazhary *et al*., 1973; Jung and Hoilat, 2021). *A. hydrophila* produced colorless colonies as reported in Park *et al*., 2011 report. Sometimes it produces dark pink colonies. In this study *A. hydrophila* produced colonies on MAC, sometimes scattered and sometimes widespread. Colony color varied from colorless pink and occasionally dark pink. This condition has been associated with bacterial gene transfer. In this study, *A. hydrophila* was found to be resistant to antibiotic enrofloxacin on MAC agar. It is a striking result that this bacteria, which is normally sensitive to MHA agar, is resistant to MAC. This situation became prominent with the use of MAC agar instead of MHA agar for the first time.

Aeromonas Isolation Base Agar is based on formulation of Ryan (Ryan, 1985). Thymol blue and bromothymol blue act as monitoring giving characteristic colony color (Himedia, 2020). *A. hydrophila* is complete *A. hydrophila* because of characteristics discovered on AIBA.

The *A. hydrophila* exhibited β-hemolytic activity on blood agar plates (Furmanek-Blaszk, 2014). In this study, *A. hydrophila* showed highly effective β-hemolysis on BA. At the same time, *A. hydrophila* produced greenish gray pigment as reported in Park *et al*., 2011 report.

The studies for antibiotic susceptibility and categorization of bacteria are carried out by international committees such as the Clinical and Laboratory Standards Institute, 2016 or the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Eucast, 2022). In the first case, breakpoints are published annually, whereas in the second case, they are permanently available and updated annually on its website (http://www.eucast.org/) (Alonso *et al*., 2017).

Dias *et al*. 2018, reported that all *Aeromonas* strains were resistant to ciprofloxacin. In contrast in his study results, *A. hydrophila* were sensitive to ciprofloxacin. This variation can be due to differences in host species and *Aeromonas* strains (Dias *et al*., 2018).

A. hydrophila has multifarious acquired and intrinsic resistance postures against antibiotics (Puzari and Chetia, 2017; Pang *et al*., 2019; Colclough *et al*., 2020; Zahedi bialvaei, 2021).

Results of this antibiotic sensitivity postures assay on *A. hydrophila* were consistent with those reported by other researchers (Trust and Chipman, 1979; Von Graevenitz and Mensch, 1968). Ciprofloxacin, enrofloxacin, gentamicin sulphamethoxazole/trimethoprim would appear to be the treatment of choice.

Antibiotic resistance profiles vary in bacteria (Byarugaba, 2004; Davis and Brown, 2016). Filik *et al*. 2021 reported that *A. hydrophila* showed multi-antibiotic resistance. Likewise, in this study, it was determined that *A. hydrophila* acquired MARI. According to the results of this research MARI was 0.33 for MHA and the MARI was 0.5 for MAC. As in other studies, it was determined that *A. hydrophila* gained MAR in this study as well.

The guardian in 2020 warned that if new antibiotics cannot be developed within the scope of research on antibiotic resistance (Anjuli, 2019), 10 million living things could be at risk from diseases each year by 2050. On the other hand, it is not the strongest or smartest who survive, but one who can adapt most to change. As Megginson (1963) pointed out, the antibiotic resistance adapted to change of environment among bacteria almost reached its peak. Today, over 20,000 potential R genes belonging to 400 different microorganisms are known, antibiotic resistance is an important problem that concerns

not only the present but also the future and threatens the whole world. The problem of antibiotic resistance of bacteria in the biofilm matrix that causes infection is one of the major problems faced (Costerton et al., 1999).

Antibiotic resistance is the greatest global threat we face today. Human and animal overuse of antibiotics is a contributing factor and major act change around antibiotic consumption is needed, but several challenges exist in communicating antibiotic resistance to the public. In 2018 UK Government relaunched television advertisement as part of the 'Keep Antibiotics Working' campaign which aimed to raise awareness of antibiotic resistance and reduce public demand for antibiotics. The findings did highlight knowledge gaps amongst research participants including vitality of completing courses of antibiotics as prescribed, and that is bacteria itself, not the person, that develops resistance (Anjuli, 2019). Valuable different results were obtained in our study in terms of differentiating the studies on this subject, which is expected to benefit from television, which is an element of reaching users in the shortest and fastest way by advertising.

As the Royal Society for Biology was forming, antibiotics resistance was being heralded as the next threat with magnitude on par with global warming. In 2016, Jim O'Neill's report was published laying out recommendations for tackling drug-resistant infections globally (Hardie, 2020).

The problem of antibiotic use is as important as other global threats. Since it is a biological problem and is absorbed into the body, it cannot be purified and causes major problems. We believe that this study will support in vivo studies and the literature on antibiotics in terms of investigating different aspects of such an important issue.

5. CONCLUSIONS

The development of the AntibiogramJ program is an important step in terms of digitization and reaching the right result. It is also important to try to see resistance capacity of *A. hydrophila* by using different agars while innovations such as AntibiogramJ program are being tried to be added to antibiotic literature.

The antibiogram assay via MHA (unalterable agar) and in this study has been adapted for use of different agars in determining antibiotic sensitivity. With the antibiogram tested on other agars instead of MHA agar, it was determined that *A. hydrophila* gained resistance on different agars to some antibiotics to which it is normally sensitive. Thus, clearer results were obtained with a different approach.

Biofilm has a very important position in antibiotic treatment. It has now been proven that bacteria in biofilm are many times more resistant to antibiotics. The use of other different agars, especially CRA, in this study also highlights resistance problems.

Antibiotic resistance of *A. hydrophila* was once again observed on different agars. The issue of using antibiotics for treatment rather than prophylactic purposes can be brought up again. İt is already prohibited in the EU the use of antibiotics for other purposes (prophylactic purposes etc.). Random use of antibiotics in drug treatment of bacterial diseases causes development of resistance. Thus, we have faced a post-antibiotic era in which our ability to challenge bacteria has diminished and the need for new strategies and approaches to deal with disease has increased.

HIGHLIGHT

1. Antibiotic resistance of *A. hydrophila* was once again observed on different agars. The issue of using antibiotics for treatment rather than prophylactic purposes can be brought up again.

2. Pathogens are very smart, random use of antibiotics in the drug treatment of bacterial diseases causes the development of resistance. Thus, we have faced a post-antibiotic era.

3. Antibiogram testing is performed on MHA agar, which is an unchangeable and unalterable rule. Here we emphasize the antibiotic resistance of other agars, similarly to the AntibiogramJ approach.

COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any studies with human participants or animals performed by any of the authors.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHİCS COMMITTEE APPROVAL

Ethics committee approval is not required for this research.

FINANCIAL DISCLOSURE

The authors have no financial disclosure to report.

AUTHOR CONTRIBUTION:

Conception/Design of study: NF
Data Acquisition: FF
Data Analysis/Interpretation: FF
Drafting Manuscript: NF
Critical Revision of Manuscript: NF
Final Approval and Accountability: NF/FF/AK
Other Technical or Material Support: NF/FF/AK
Supervision: NF/AK

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