Fishery Technology 57 (2020) : 121 - 127

Haemolytic studies on Fish, Sheep and Human Blood Agar against Pathogenic Bacteria

Ranjit Kumar Nadella*, Minimol V. A., Ezhil Nilavan, T. Muthulakshmi, K. Ahamed Basha and M. M. Prasad

ICAR-Central Institute of Fisheries Technology, Willingdon Island, P. O. Matsyapuri P. O., Cochin - 682 029, India

Abstract

In this study, β-haemolytic activity of fish blood (*Cyprinus rubrofuscus*), sheep blood (SB) and human blood (HB) was assessed for studying haemolysis against reference bacterial strains *Edwardsiella tarda* (ATCC 15947); *Aeromonas hydrophila* (ATCC 35654); *Enterobacter cloacae* (ATCC 13047); *Pseudomonas aeruginosa* (ATCC 10145); *Escherichia coli* (ATCC 35218); *Staphylococcus aureus* (ATCC 43300); *Vibrio cholera* (MTCC 3906) and *Salmonella paratyphii* (ATCC 15305). Both human blood and fish blood showed similar β-haemolytic activity for all pathogens except *E. cloacae* on fish blood. The zone of haemolysis (mm) recorded in FB is 18, 28, 0, 22, 25, 14, 20, 22; in HB is 13, 15, 14, 23, 17, 18, 15, 17 and in SB is 14.6, 13.8, 15.8, 26.2, 19.4, 21.2, 17.6, 16.2 after 48 h respectively for *E. tarda*; *A. hydrophila*; *E. cloacae*; *P. aeruginosa*; *E. coli*; *S. aureus*; *V. cholera* and *S. paratyphii*. The present study also reports the antimicrobial property of fish blood against the commonly occurring fish pathogens. The result indicated a decrease in optical density readings in fish blood supplemented broth compared to control broth (without blood). This indicates clear inhibition of growth for the three pathogens in BHI broth supplemented with fish blood possesed due to the presence of antimicrobial substances in the fish blood. In conclusion, β-haemolytic activity observed better in FB plates when compared with HB plates and therefore, fish blood can be used as an alternative to sheep blood for haemolytic studies of fish pathogens.

Received 17 March 2020; Revised 25 March 2020; Accepted 02 April 2020

**E-mail: nranjeetkumar@gmail.com*

Keywords: Haemolysis, Fish blood, Pathogens, Antibacterial activity

Introduction

Bacterial diseases have become major impediments to aquaculture especially in case of finfish culture. Bacterial species belonging to at least 13 genera have been reported to be pathogenic to aquatic animals include Gram-negative bacteria such as *Aeromonas, Edwardsiella, Vibrio, Pseudomonas,* and Gram-positive namely *Staphylococcus* (Klesius et al., 2011). They can cause mortality to fishes resulting in loss to farmers. The annual economic loss to the aquaculture industry due to diseases is runs into billions of US dollars worldwide. Worlds over, in aquaculture losses due to diseases were estimated by World Bank approximately each year around 3 billion US $ (Subasinghe et al., 2001).

Edwarsiellosis is caused by *Edwardsiella* spp is an acute or chronic bacterial disease affecting several fish species (Park et al., 2012). Motile aeromonad septicaemia (MAS) is caused by *Aeromonas hydrophila* which is a gram negative bacteria causing heavy mortalities in cultured fishes worldwide (Fang et al., 2004; Xia et al., 2004). *Pseudomonas* is an opportunistic pathogen that has been reported to cause disease in a number of fish species when the fish comes under stress (Bullock, 1965). Food borne pathogens are responsible for the disease outbreaks around the world. Among them the outbreaks of *V. cholerae* is seen when there is water contamination due to faeces from infected persons or the contamination of aquatic food products with the contaminated water serves as a source of infection (Rabbani et al., 1999). The bacterium responsible for causing gastroenteritis in humans is *Escherichia coli.* The fish and fishery products that are not handled properly leads to outbreak with pathogenic *E. coli* (Murugadas

et al., 2016). In the recent years several outbreaks associated with *Staphylococcus aureus* is recorded which a multidrug resistant bacterium causing heavy morbidity and is difficult to treat once the infection occurs (Sivaraman et al., 2016). *Salmonella* is considered as a major foodborne pathogen that is frequently associated with the outbreaks in fishery products. Therefore, the presence of this bacterium in aquatic environments serves as major reservoirs (Kumar et al., 2009).

Rapid detection of pathogenic bacteria is carried out using several techniques. Among them, haemolysis is considered as one of the striking feature for the detection of the pathogenicity of the bacteria and has gained importance in the fish disease diagnostics. Haemolysis is defined as release of hemoglobin along with intracellular components from lysed erythrocytes into plasma of the cells due to damage of the cell membrane. The alpha, beta, and gamma types of haemolysis are the common types of haemolysis found in blood agar (Paulson & Brown, 1932). Hoppe & Schlangenhauf, (1989) compared growth of *Bordetella pertussis* on three kinds of blood agar plates *i.e*., defibrinated sheep blood, horse blood, and human blood and concluded growth was better on plates supplements with horse blood. Gratten et al. (1994) compared horse and goat blood in blood agar as supplements for isolation of human pathogenic bacteria (*Streptococcus pneumonia* and *Haemophilus influenza*). Santos et al. (1999) studied haemolytic activity of different *Aeromonas* strains isolated from fish for beta-hemolytic activity on Columbia agar base supplemented with rabbit erythrocytes and sheep erythrocytes. Anand et al. (2000) studied haemolysis of pathogenic bacteria *viz*., *Candida albicans*, *Enterococcus* sp. and *Haemophilus* species on blood agar plates prepared from blood of pig and goat as substitutes for sheep blood. Alavandi & Ananthan (2003) reported that the environmental strains of *Aeromonas* sp. produced distinct β-haemolysis compared to the clinical isolates on blood agar plates.

Russell et al., 2006 studied citrated sheep blood agar as an alternative to citrated human blood as a bacterial culture medium for studying haemolysis. Egwuatu et al. (2014) studied the growth and morphology of pathogenic bacteria on sheep and horse blood. Gattu et al. (2017) analysed the growth of *Helicobacter Pylori* on the blood agar plates made of goat (Caprine), Sheep (Ovine), Chicken (Gallus) and fish. The studies carried out on haemolysis using fish blood are scant. Pier et al. (1978) used blood from freshwater dolphin for carrying of haemolysis test against *Streptococcus iniae*. Eldar et al., 1995 isolated sixty Streptococcal strains from diseased fish using blood from sheep and fish. Dhevendaran & Georgekutty (1998) screened pathogenic *Vibrio* spp., from fish using blood agar incorporated with fish blood. Pasnik et al. (2005) studied four piscine bacterial isolates (*Streptococcus iniae*, *Staphylococcus epidermidis, Edwardsiella tarda* and *Acinetobacter calcoaceticus*) for their haemolytic activity on blood agar plates prepared by using blood collected from Nile tilapia, *Oreochromis niloticus*. The present study reports comparison of haemolysis on fish, sheep and human blood agar plates against human and fish pathogens.

Materials and Methods

The species used for the present experiment was freshwater ornamental fish, *Cyprinus rubrofuscus* commonly called as Koi carp. The ornamental fish with an average of weight of 50-60 g was brought form a local ornamental fish hatchery in Ernakulam district. Immediately after reaching the laboratory, the fish was disinfected by dipping in 50 ppm $KMNO_4$ and carefully transferred to an aquarium tank (100 L). Fish were acclimatized to captive conditions for a period of 7 days to reduce the stress and commercial feed was given twice in a day at 3% body weight for one week. The water parameters maintained are temperature at 30ºC, pH-7.2 and continuous aeration is provided.

The reference strains employed were; *Edwardsiella tarda* (ATCC 15947); *Aeromonas hydrophila* (ATCC 35654); *Enterobacter cloacae* (ATCC 13047); *Pseudomonas aeruginosa* (ATCC 10145); *Escherichia coli* (ATCC 35218); *Staphylococcus aureus* (ATCC 43300); *Vibrio cholera* (MTCC 3906) and *Salmonella paratyphii* (ATCC 15305). Strains were revived and sub cultured from glycerol stock under aseptic conditions. Purification and biochemical identification of the cultures were carried out as per standard methods mentioned in Bergy's Manual (Brenner et al., 2004).

Before the collection of blood, the fish was anesthetized with clove oil in order to reduce the stress. Blood was withdrawn from the anesthetized fish ventral vein (Vena caudalis) using a 26 guage syringe rinsed with 2.7% EDTA solution. The collected blood was transferred into another tube

coated with EDTA powder (as an anticoagulant) and shaken well in-order to prevent the haemolysis.

Fresh human blood was collected from healthy persons with the addition of anticoagulant (heparin) in the blood collection tubes and labeled properly. Blood was collected from fish and humans by following standard ethical procedures. Ready-made agar plates incorporated with 5% sheep blood used in the present study was procured from Himedia, Mumbai, India.

The composition of basal medium used for the preparation of blood agar is follows: Bactopeptone-10 gm; Sodium chloride -5 gm; Bactoagar- 18 gm. Basal agar medium was prepared by mixing the above components in 1000 ml distilled water. The media was sterilized at 121ºC for 15 min. The basal medium was cooled to 50ºC before adding to the plates. The freshly collected bloods were added to the cooled medium and rotated for complete mixing. Then the medium is slowly poured into the plate and allow it to solidify. After solidification, the plates were left at room temperature to check the sterility of the medium. Later, the plates were stored at 4ºC for one week.

Freshly grown cultures on TSA slants were taken with the loop and dissolved with normal saline (0.85% NaCl). The bacterial concentration was adjusted to 0.5 ($OD_{(650)}$. Then serial dilutions were made up to 10^{-9} with sterile normal saline. Then 25 µl from 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9} dilutions were spot inoculated onto freshly prepared fish, sheep and human agar plates in duplicates. Bacterial cultures were observed for β haemolytic activity after 24 and 48 h of incubation at 28ºC and zone of inhibition (mm) was measured.

The antibacterial activity of the fish blood against the fish pathogens *E. tarda, A. hydrophila* and *P. aeruginosa* was carried out using spectrophotometer (turbidometric method) (Fig. 1). Bacterial suspension (OD_{600} adjusted to 0.5 corresponds to 1 X lO^6 cfu ml^{-1}) was prepared and added to the BHI broth (20 ml). The freshly collected fish blood (1%) was added to the broth. Two control sets were used; one for control bacteria incubated with equal volume of fish blood and BHI broth and another, bacteria without blood suspended in BHI broth. Incubations were carried at 30°C in the incubator. The bacterial growth was monitored in all the three tubes using spectrophotometer at 4, 8, 16, 24 and 48 h.

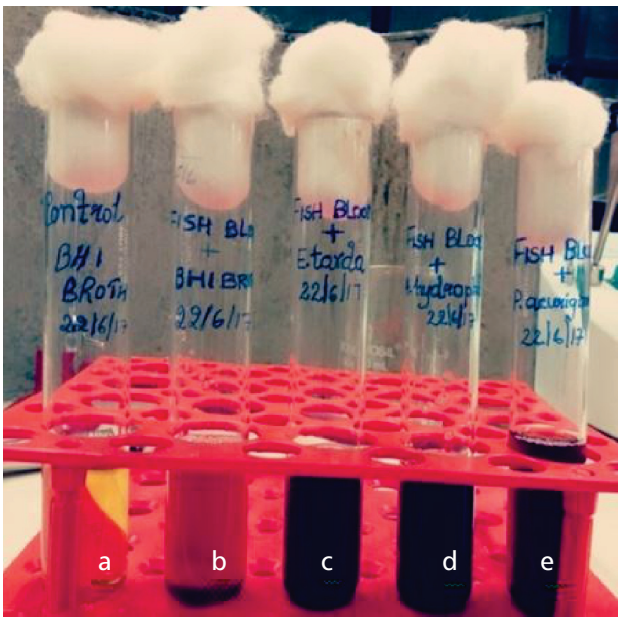

Fig. 1. Image showing control broth (a), broth supplemented with fish blood (b), broth supplemented with fish blood and inoculated with bacteria viz., *E. tarda* (c), *A. hydrophila* (d) and *P. aeruginosa* (e) for spectrophotometer reading

Results and Discussion

Among the dilutions plated on different blood agar plates, prominent haemolysis was observed on all blood agar plates in 10^{-3} dilution of respective bacterial cultures which were then selected for measuring the zone of heamolysis. The diameter of the zone of haemolysis on fish, sheep and human blood agar plates after 24 h and 48 h is given in Table 1. For fish pathogenic bacteria maximum zone was observed for *A. hydrophila* (28 mm) followed by followed by *P. aeruginosa* (26.2) and *E. tarda* (18 mm) on fish blood agar base. Similarly, for food borne pathogens maximum zone was observed on sheep blood agar base for *E. coli* (25 mm), followed by *S. aureus* (22.2 mm). However, no zone of haemolysis was found for *E. cloacae* on fish blood agar base. Clear β-Haemolysis observed on blood agar plates supplemented with fish blood (Fig. 2), sheep blood (Fig. 3) and human blood agar (Fig. 4).

Haemolysis is one of the striking features for the detection of the pathogenicity of the bacterial pathogens (Liefson, 1932) and has gained importance in the fish disease diagnostics. Haemolysis is caused by certain lytic enzymes produced by the pathogenic bacteria which act on the red blood cells

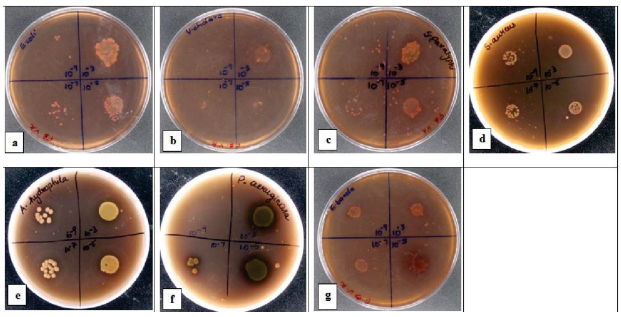

Fig. 2. β-Haemolysis observed on fish blood agar plates spot inoculated with different bacteria viz., *E. coli* (a), *V. cholera* (b), *S. paratyphii* (c), *S. aureus* (d), *A. hydrophila* (e), *P. aeruginosa* (f) and *E. tarda* (g)

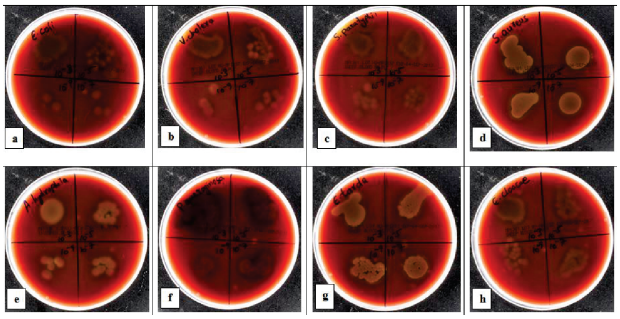

Fig. 3. β-Haemolysis observed on sheep blood agar plates spot inoculated with different bacteria viz., *E. coli* (a), *V. cholera* (b), *S. paratyphii* (c), *S. aureus* (d), *A. hydrophila* (e), *P. aeruginosa* (f) *E. tarda* (g) and *E. cloacae* (h)

present in blood agar leading to lysis. Fish blood also contains several antibacterial proteins that play very important role in the immune responses against bacterial invasion. The innate immune system garnered lesser attention when compared to higher vertebrates. In the present study, fish blood was used to confirm and evaluate its performance

Table 1. Diameter of haemolysis zone (mm) on human and fish blood plates against the fish and food borne pathogens. Values represent mean of readings from blood agar plates.

Sl. No		Diameter of haemolysis zone (mm)					
		Fish blood		Sheep blood		Human blood	
	Culture	24 h	48 h	24 h	48 h	24 h	48 h
1	*E. tarda*	9.6	18.0	7.4	14.6	6.5	13.0
2	*A. hydrophila*	16.5	28.0	7.0	13.8	7.6	15.0
3	*E. cloacae*	0	0	8.0	15.8	7.1	14.0
4	*P. aeruginosa*	13.5	22.0	12.4	26.2	11.9	23.0
5	*E. coli*	17.4	25.0	11.6	19.4	9.6	17.0
6	*S. aureus*	7.6	14.0	12.6	21.2	11.8	18.0
7	*V. cholera*	12.5	20.0	11.0	17.6	10.0	15.0
8	*S. paratyphi*	17.8	22.0	10.2	16.2	11.6	17.0

Table 2. Antibacterial activity recorded in control and fish blood enriched broth against fish pathogens. Values represent mean of spectrophotometer readings (OD_{600} nm)

	Antibacterial activity of fish blood (OD_{600} nm)					
Time interval	Control broth			Fish blood enriched broth		
	A. hydrophila	*P. aeruginosa*	*E. tarda*	*A. hydrophila*	*P. aeruginosa*	*E. tarda*
0h	0.50	0.50	0.50	0.50	0.50	0.50
4h	1.26	0.81	1.03	0.68	0.51	0.60
8h	1.50	1.17	1.74	1.27	0.73	0.90
16h	1.72	1.54	1.97	1.49	0.91	1.23
24h	2.15	1.73	2.13	1.49	0.95	1.25
48h	2.38	1.93	2.16	1.54	1.02	1.32

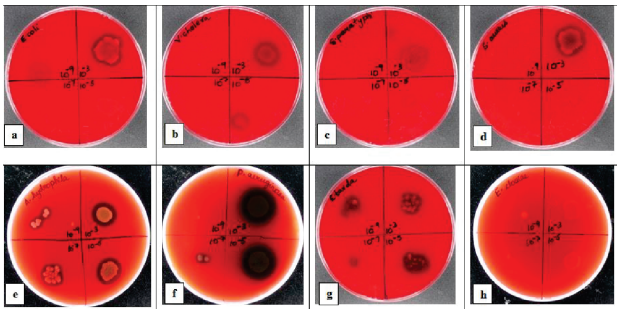

Fig. 4. β-Haemolysis observed on human blood agar plates spot inoculated with different bacteria viz., *E. coli* (a), *V. cholera* (b), *S. paratyphii* (c), *S. aureus* (d), *A. hydrophila* (e), *P. aeruginosa* (f) *E. tarda* (g) and *E. cloacae* (h)

as an alternate source of supplement for the preparation of blood agar for haemolysis test and also reports the antimicrobial property of fish blood on the commonly occurring fish pathogens. Results observed during this study were compared with haemolytic activity of same cultures on sheep and human blood (HB). Fish, sheep and human blood showed similar β-haemolytic activity for all pathogens except, *E. cloacae* which did not show haemolysis on fish blood. The results showing haemolysis of fish blood was similar to hemolysic studies conducted by Pasnik et al. (2005). He reported haemolytic activity of four piscine bacterial isolates on blood agar plates prepared using blood from Nile tilapia, *Oreochromis niloticus*. Similarly, haemolytic studies on blood agar using aquatic animals blood was also carried out against pathogenic bacteria (Pier et al., 1978; Eldar et al., 1995; Dhevendaran & Georgekutty, 1998; Gattu et al., 2017). In other haemolytic studies the blood collected from several animals was used as an alternative to sheep or human blood as a supplement in blood agar for haemolytic studies (goat, sheep, chicken) (Egwuatu et al., 2014); defibrinated horse blood (Hoppe & Schlangenhauf, 1989); goat and horse blood (Gratten et al., 1994); pig and goat blood (Anand et al., 2000) and rabbit erythrocytes (Santos et al., 1999). In our study, zone of haemolysis observed was on fish blood agar plates for aquatic pathogens compared to sheep and human blood gar plates suggest that the host specificity of the bacteria. Similarly Pasnik et al. (2005) reported zone of haemolysis of the pathogenic bacteria on fish blood agar plates prepared from Nile tilapia.

The antibacterial activity of fish blood against the fish pathogens (*A. hydrophila, E. tarda* and *P. aeruginosa*) was carried out by using spectrophotometric (turbidometric) method. The activity observed against the fish pathogens is shown in Table 2. In compare to control both, blood enriched broth showed lesser optical density indicating the antibacterial activity against the pathogens.

The antimicrobial property of fish blood was determined by using reference cultures *Pseudomonas aeruginosa*, *Edwardsiella tarda* and *Aeromonas hydrophila*. The clear inhibition of growth was noticed for the three pathogens in BHI broth supplemented with fish blood when compared to control broths. The presence of antimicrobial substances in the fish blood is responsible for the inhibition of growth. The survival rate of *S. aureus* in a standard volume of defibrinated blood to estimate the bactericidal power of blood was studied by Miles & Mishra, (1938). Krumweide & Kutter, 1938 concluded that inhibitory substances that are present in blood of animals suppressed the growth of influenza organisms. Villamil et al. (2002) reported that ECPs produced by *L. lactis* were significantly reduced the growth of the pathogenic *V. anguillarum*.

The inhibition factors present in the fish blood are essential to prevent bacterial growth (Yano, 1996; Ellis, 1999; Salinas et al., 2011). Main factors responsible are transferrin, which shows higher affinity towards iron which is needed for the bacterial growth (Bullen & Griffiths, 1987; Stafford & Belosevic, 2003). Lectins, which can bind to the carbohydrate molecules present on the bacterial cell wall and cause agglutination (Arason, 1996). Lysines which are mainly found in body tissue and blood attacks the cell membranes of the pathogenic bacteria and cause lysis (Ellis, 2001). The peptidoglycan layer present in the bacterial cell wall of Gram-negative bacteria and Gram-positive is lysed by the-lysozyme (Murray & Fletcher, 1976). C-reactive protein also pays a role in inhibiting the bacterial growth by binding to phosphorylcholine of the microorganisms besides participating in phagocytosis (Magnadottir, 2011) and complement activation (Nakanishi et al., 1991). In this study too, the presence of these substances in fish blood might have played a role in inhibiting the bacterial growth in the broth supplemented with fish blood. Accordingly, marked decrease in optical density was noticed when compared to control broth. The presence of antimicrobial substances in the fish blood is responsible for the inhibition of growth.

This warrants further investigations to purify, identify and characterize these substances from fish blood.

In summary, the studies on haemolytic activity of important fish and human pathogenic bacteria revealed that the β-haemolytic activity observed better in FB plates when compared with SB and HB plates and therefore, fish blood can be used as an alternative to sheep blood for haemolytic studies of fish pathogens. Higher zone of haemolysis for aquatic pathogens on fish blood agar suggest the host specificity of bacterial pathogens. Therefore, fish blood can be employed as an alternate source to sheep blood for studying haemolysis of fish pathogenic bacteria.

Acknowledgments

Authors are grateful to the Indian Council of Agricultural Research, New Delhi, India, for the financial support and to the Director, ICAR-Central Institute of Fisheries Technology, Cochin, India, for providing all the facilities required for the present study.

References

Alavandi, S. V. and Ananthan, S. (2003) Biochemical characteristics, serogroups, and virulence factors of Aeromonas species isolated from cases of diarrhoea and domestic water samples in Chennai. Indian. J. Med. Micrbiol. 21(4): 233-238

Anand, C. R., Gordon, A., Shaw, H., Fonseca. K. and Olsen, M. (2000) Pig and goat blood as substitutes for sheep blood in blood supplemented agar media. J Clin Microbiol 38: 591-594

Arason, G. J. (1996) Lectins as defence molecules in vertebrates and invertebrates. Fish Shellfish Immunol. 6: 277-289

Bullen, J. J. and Griffths, E. (1987) Iron and infection - Molecular, physiological and clinical aspects. Chichester: Wiley. 515 p

Bullock, G. L. (1965) Characteristics and pathogenicity of a capsulated *Pseudomonas* isolated from goldfish. Appl. Microbiol. 13: 89-92

Dhevendaran, K. and Georgekutty, M. I. (1998) Production of extracellular enzymes by pathogenic *Vibrio* spp. in fish. Fishery Technol. 35(2): 99-104

Egwuatu, T. O., F. T., Ogunsola, I. M., Okodugha, O. B., Jide, D. G., Arewa and O. A. Osinupebi (2014) Effect of blood agar from different animal blood on growth rates and morphology of common pathogenic bacteria. Adv. Microbiol. 4: 1237-1241

Eldar, A., Bejerano, Y., Livoff, A. Horovitcz, A. and H. Bercovier (1995) Experimental *Streptococcal* meningo-encephalitis in cultured fish. Vet. Microbiol., 43: 33110

Ellis, A. E. (1999) Immunity to bacteria in fish. Fish Shellfish Immunol. 9: 291-308

Ellis, A. E. (2001) Innate host defense mechanism of fish against virus and bacteria. Dev. Comp. Immunol. 25: 827-839

Fang, H. M., Ge, R and X Sin, R.M. (2004) Cloning, characterization and expression of *Aeromonas hydrophila* major adhesin. Fish Shellfish Immunol. 16: 645-58

Gratten, M., Battistutta, D., Torzillo, P., Dixon, J. and K. Manning (1994) Comparison of goat and horse blood as culture medium supplements for isolation and identification of *Haemophilus influenzae* and *Streptococcus pneumoniae* for upper respiratory tract secretions. J. Clin. Microbiol., 32: 2871-2872

Gattu, S., Prabhusaran, N., Sridharan, K. and Kayalvizhi, N (2017) Comparative analysis of the growth of *Helicobacter pylori* in different blood agar medium. Mint. J. Pharmaceut. Med. Sci., 6(3): 23-25

Hagi, T. and Hoshino, T. (2009) Screening and characterization of potential probiotic lactic acid bacteria from culture common carp intestine. Biosci. Biotechnol. Biochem. 73: (80746) 1-5

Hoppe, J. E. and Schlangenhauf, M. (1989) Comparison of three kinds of blood and two incubation atmospheres for cultivation of *Bordetella pertussis* on charcoal agar. J. Clin. Microbiol. 27: 2115-2117

Klesius, P. H. and Pridgeon, J. W. (2011) Live attenuated bacterial vaccines in aquaculture. In: Proceedings of the 9th international symposium on Tilapia in aquaculture. 18-26 pp

Krumweide, E. and Kutter, A. G. (1938) A growth inhibitory substance for the influenza group of organisms in the blood of various animal species. The use of the blood of various animals as a selective medium for the detection of hemolytic *Streptococci* in throat cultures. J. Exp. Med. 67: 429-441

Kumar, R., Surendran, P. K. and Thampuran, N. (2009) Distribution and genotypic characterization of *Salmonella* serovars isolated from tropical seafood of Cochin, India. J. Appl. Microbiol. 106(2): 515-524

Leifson, E. (1932) Types of bacteria on blood and chocolate agar and the immediate cause of these types. J. Bacteriol. 24(6): 473-487

Magnadottir, B., Audunsdottir, S. S. and Bragason, B. T. H. (2011) The acute phase response of Atlantic cod (*Gadus morhua*): Humoral and cellular responses. Fish Shellfish. Imunol. 30: 1124-1130

Miles, A. A. and Misra, S. S. (1938) The Estimation of the bactericidal power of the blood. J. Hygiene, 38: 732-749

Mishra, S., Balakrish, G., Bhadra, K. R., Sikdar, N. S. and Pal, C. S. (1987) Comparison of selective media for primary isolation of *Aeromonas* species from human and animal feaces. J. Clin. Microbiol. 25(11): 2040-2043

Murray, C. K. and Fletcher T. C. (1976) The immune histochemical localization of lysozyme in plaice (*Pleuronectes platessa* L) tissues. J. Fish Biol. 9: 329-334

Murugadas, V., Joseph, T. C. and Lalitha, K. V. (2016) Distribution of pathotypes of *Escherichia coli* in seafood from retail markets of Kerala, India. Indian J. Fish. 63(1): 152-155

Nakanishi, Y., Kodama, H. and Murai, T. (1991) Activation of rainbow trout complement by C-reactive protein. Am. J. Vet. Res. 52: 397-401

Park, S. B., Aoki, T. and Jung, T. S (2012) Pathogenesis of and strategies for preventing *Edwardsiella tarda* infection in fish. Vet. Res. 43(1): 67

Pasnik, D. J., Evans, J. J., and Klesius, P. H. (2005) Nile tilapia, *Oreochromis niloticus*, blood agar and the culture of fish bacterial pathogens. Bull. Eur. Ass. Fish Pathol. 25 (5): 221-227

Paulson, M. and Brown, J. H. (1932) The appearances produced in blood agar by bacteria of the Bacterium coli group. Jour. Inf. Dis. 51: 407-411

Pier, G. B., Madin, S. H. and S. Al-Nakeeb (1978) Isolation and characterization of a second isolate of *Streptococcus iniae*. Int. J. Sys. Bacteriol. 28, 311-314

Rabbani G. H. and Greenough, W. B. (1999) Food as vehicle of transmission of cholera. J. Diarhoeal. Dis. Res. 17: 1-9

Russell, F. M., Biribo, S. S. N., Selvaraj, G., Oppendisano, F., Wearren, S., Seduadua,A., Mulholland, E. K. and J. R. Carapetis (2006) As a bacterial culture medium, citrated sheep blood agar is a practical alternative to citrated human blood agar in laboratories in developing countries. J. Clin. Microbiol. 44: 3346-3351

Salinas, I., Zhang, Y. A. and Sunyer, J. O. (2011) Mucosal immunoglobulins and B cells of teleost fish. Dev. Comp. Immunol. 35: 1346-1365

Santos, J. A., Gonzalez, C. J., Otero, A. and Garcia-Lopez, M (1999) Hemolytic activity and siderophore production in different *Aeromonas* species isolated from fish. Appl. Environ. Microbiol. 65: 5612-5614

Stafford, J. L. and Belosevic, M. (2003) Transferrin and the innate immune response of fish: identification of a novel mechanism of macrophage activation. Dev. Comp. Immunol. 27: 539-554

Sivaraman, G. K., Deesha, V., Prasad, M. M., Jha, A. K., Visnuvinayagam, S., Nadella, R. K., Chandni, V and Basha, A (2016) Incidence of community acquired methicillin-resistant *Staphylococcus aureus* (CA- MRSA) in seafood and its environment, Gujarat, India. Int. J. Recent Sci. Res. 7(11): 14279-14282

Subasinghe, R. P., Bondad-Reantaso, M. G. and McGladdery, S. E. (2001) Aquaculture development, health and wealth. Subasinghe, R.P., P. Bueno, M. J. Phillips, C. Hough, S. E. McGladdery and J. R. Arthur, eds. Aquaculture in the Third Millennium. Technical Proceedings of the Conference on Aquaculture in the Third Millennium, Bangkok, Thailand, 20-25 February 2000. pp. 167-191. NACA, Bangkok and FAO, Rome

Villamil, L., Tafalla, C., Figueras, A., and Novoa, B. (2002) Evaluation of immunomodulatory effects of lactic acid bacteria in Turbot (*Scophthalmus maximus*). Clin. Diag. Lab. Immunol. 9(6): 1318-1323

Xia, C., Ma, Z., Rahman, H. and Wu, Z (2004) PCR Cloning and identification of the b-hemolysin gene of *Aeromonas hydrophila* from freshwater fishes in China. Aquacult. 229: 45-53

Yano, T. (1996) The nonspecific immune system: Humoral defence. In: Iwama, G.; Nakanishi, Eds. The Fish Immune System: Organism, Pathogen and Environment. San Diego, Academic Press. pp 105-157