ejpmr, 2018,5(6), 50-54

SJIF Impact Factor 4.897

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

THE EFFECT OF SOME PATHOGENIC BACTERIAL PROTEASES ON AVIAN INFLUENZA VIRUSES H5N1 AND H9N2

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Article Received on 26/03/2018 **Article Revised on 16/04/2018** **Article Accepted on 06/05/2018**

ABSTRACT

Microbial proteases are responsible for proteolytic cleavage activation of Influenza A Virus (H5N1 and H9N2) hemagglutinin (HA). We examined the proteolytic activities of some pathogenic bacterial isolates such as *Staphylococcus aureus, Pseudomonas aeruginosa* and *Mycoplasma gallisepticum.* The contribution of endogenous and microbial proteases (exogenous proteases) in the avian influenza pathogenicity were studied by inoculation of three groups of embryonated chicken eggs (SPF). The first group was inoculated simultaneously with either viruses; H5 and H9, with different concentrations of bacterial proteases. The second group was inoculated with pre-incubated viruses with different concentrations of the bacterial proteases. The third group was control (virus only, protease only and PBS). An increase in viruses' titer was observed in eggs inoculated with virus treated with microbial proteases incomparable to that inoculated with untreated viruses. These results confirmed the synergism between influenza virus and pathogenic bacteria based on proteolytic activation of the hemagglutinin by bacterial proteases. Further, H9N2 virus titer was significantly increased as compared to H5N1 virus titer in eggs with prominent increase of H9N2 virulence that was noted in the death time of the eggs reaching that of H5N1. *M. gallisepticum* showed an increase in virus titer but lower than that caused by *Staphylococcus aureus and Pseudomonas aeruginosa* revealing the difference between serine and cysteine proteases in cleavage activation of influenza virus HA.

KEYWORDS: S. Aureus, P. Aeruginosa, M. Gallisepticum, Proteases, AIV.

INTRODUCTION

Morbidity and mortality caused by influenza virus infection usually associates with secondary bacterial complications (Morens et al., 2008, Lee et al., 2010). Staphylococcus aureus is the most frequent secondary invader to cause fatal pneumonia in many parts of the world, compared to other circulating strains, probably related to altered expression or regulation of particular bacterial virulence factors, such as cytotoxins or adherence factors (McCullers et al., 2014). Chronic respiratory tract infections caused by Pseudomonas aeruginosa are one of most difficult infections to control and often develop acute severity with viral and/or bacterial superinfection (Seki. et al., 2004). Mycoplasma gallisepticum is considered as a circulating respiratory pathogen in poultry flocks contributing to multi-infections with other respiratory pathogens such as influenza virus (Sid et al., 2016). Influenza viruses have two surface glycoproteins; hemagglutinin (HA) and neuraminidase (NA). HA binds to the cell receptor via sialic acid residues on the surface of host cells and mediates membrane fusion (Skehel and Wiley, 2000). NA is a receptor- destroying enzyme that binds and cleaves sialic acid on the cell surface allowing efficient release of virus particles (Mitnaul et al., 2000). Influenza virus requires activation before infection, and activation efficiency is an important determinant of viral virulence (Bosch et al., 1981; Klenk et al., 1994; Chen et al., 1998). The process of virus activation involves proteolytic cleavage of HA. After cleavage, the precursor protein HA0 is separated into HA1 and HA2, which is required for successful viral entry. Thereafter, the virus binds to the host surface, internalized by receptor-mediated endocytosis and deposited into the endosomal compartment (Skehel and Wiley, 2000). The Proteases for HA activation are not limited to host cell proteases, bacterial proteases are also involved in this process (Bottcher et al., 2013). For instance, staphylococcus sp. secretes a soluble protease that is able to activate HA (Scheiblauer et al., 1992). Furthermore, indirect activation of influenza HA by bacterial co-factors is also a possible way of HA activation. Pseudomonas aeruginosa also indirectly activates HA by activating host proteases (Scheiblauer et al., 1992). Thus, here we aimed to test the effect of different bacterial proteases on H5N1 and H9N2 avian influenza viruses circulating in Egypt.

MATERIALS AND METHODS

Bacteria

Staphylococcus aureus and *Pseudomonas aeruginosa* were a kind gift from National Institute of Oceanography and Fisheries (NIOF), Alexandria, Egypt. Stock cultures were maintained at 4°C on nutrient agar slants (Malash et al., 2016).

Mycoplasma gallisepticum was obtained from Animal Health Institute, Shalateen branch, Egypt. The stock culture was maintained in Frey's broth (Sid et al., 2016).

Viruses

Avian influenza viruses; H5N1 and H9N2 were isolated from clinically infected poultry (Nahla et al., 2018). Viruses were propagated in 11-day old chicken embryos for 48 h at 37°C; the virus titer was calculated by hemagglutination (HA) test according to OIE terrestrial manual (2015).

Cultivation of bacteria for protease production

For detection of protease production, *S. aureus* and *P. aeruginosa* were cultured on Luria birtani (LB) broth (Farhat et al., 2008) and incubated at 37℃ for 24 hrs with shaking at 250 rpm (Bajaj and Jamwal, 2013). *Mycoplasma gallisepticum* was cultured on Frey's broth (Sid et al., 2016) and incubated at 37℃ for 12 days with shaking at 250 rpm till the broth turned from red to yellow color indicating growth of *M. gallisepticum*.

For purification of protease, the bacterial culture broth was centrifuged at 12,000 x *g* for 10 min at 4°C and the supernatant was filtered through 0.22-µm filter according to Callan et al., (1997). The filtrate was stored in 1ml aliquots at -20℃.

Screening for proteolytic activity in the bacterial supernatants

The culture supernatants were tested for proteolytic activity using casein agar (Mancini et al., 2008) and skim milk agar (0.1%, 1%, 2.8% and 5%) according to Shuva et al., (2015). The culture supernatants were placed in 3-mm-diameter wells cut in the agar and incubated at 37°C for 24-48 hrs according to Shuva et al., (2015). The proteolytic activity was noted as a clear zone around the sample well.

Table 1: The composition of different substrate concentrations in test agar.

Type of medium	Casein agar 0.6%	Skim milk 5%	Skim milk 0.1 %	Skim milk 1%	Skim milk 2.8%
Composition of each medium	-Casein 0.6% w/v -Tris Hcl 25Mm -Nacl 150Mm -Agar 1.5%	-Peptone 0.1% -Skim milk 5% w/v -Nacl 0.5% w/v -Agar 2%	-Peptone 5 g/l -Yeast extract 2.5 g/l -Dextrose 1g/ -Skim milk 1g/l -Agar 12.5g/l	-Skim milk powder 10 g/l -Agar 15g/l	-Skim milk 28g/l -Casein enzymic hydro lysate 5g/l -Yeast extract1g/l -Dextrose1g/l -Agar 15 g/l

Protease assay

Protease activity of the bacterial supernatant was determined by the method described by Shuva et al., (2015).

The effect of bacterial supernatant on influenza virus infectivity

This was done to detect the cleavage activation and inactivation of the treated virus by bacterial supernatant. H5N1 and H9N2 viruses were diluted in phosphate buffer saline (PBS) to a titer of 2^4 and 2^3 HA units /ml, respectively. Eleven-day old embryonated chicken eggs (ECE) were grouped into three groups in triplicates. The first group was simultaneously inoculated with each virus followed by the different proteases at three different concentrations (50 unit/ml, 100 unit/ml and 150 unit/ml). The second group was inoculated with the virus pre-incubated with the different proteases at three different concentrations (50 unit/ml, 100 unit/ml and 150 unit/ml). The third group was a control group comprising eggs inoculated with proteases only, each virus only and PBS. Post inoculation, the eggs were incubated at 37°C and monitored daily. The allantoic fluid was collected for virus titration by HA test according to Mancini et al., (2005).

RESULTS

Screening for protease production

The influence of different concentrations of the substrate on screening of protease production by the *bacterial isolates* was assessed by Casein agar diffusion test. Five different concentrations of the culture media were tested (Table 1). It was observed that the well-developed zones were formed on 1% skim milk agar medium (Figure.1). The observed clear zones were 15, 18 and 14 mm in diameter for Staphylococcus aureus, Pseudomonas aeruginosa and Mycoplasma gallisepticum, respectively.

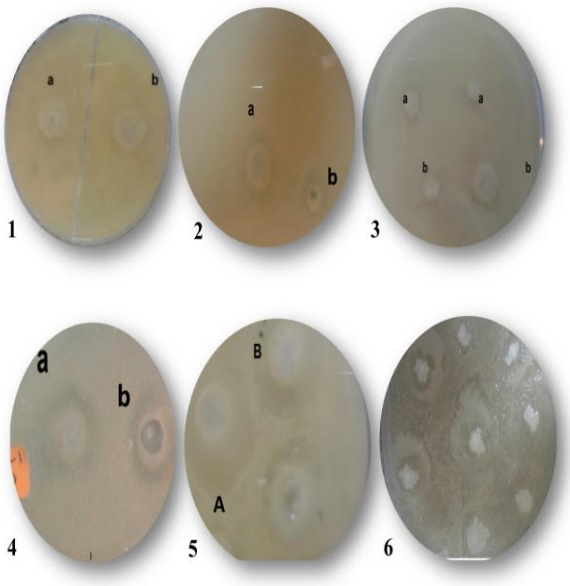

Fig.1. Screening of protease production. (1) Casein agar medium showed ill-developed zones (1-1.5 mmØ). (2) 5% skim milk agar showed ill-developed zones (2-2mmØ). (3) 2.8%skim milk agar showed zones of 3-3.5mmØ. (4) 0.1% skim milk agar showed clear zones of 5-6mmØ. (5) 1% skim milk agar showing A; *P.aeruginosa*, and B; *S.aureus* with 15 and 18 mmØ, respectively. (a) *Pseudomonas aeruginosa* (b) *Staphylococcus aureus*. (6) Mycoplasma gallisepticum on 1% skim milk agar showing a clear zone of 14mmØ.

Protease assay

The purified bacterial supernatant was assessed in biochemical analyser using end-point method to measure the protease concentration. The protease concentrations were 201.48, 203.86 and 182.65 unit/ml for *S. aureus*, *P. aeruginosa* and *M. gallisepticum*, respectively.

The effect of bacterial supernatants on influenza virus infectivity

Three different concentrations of protease (50, 100, 150 u/ml) produced by different bacterial isolates were inoculated simultaneously or pre-incubated with H5N1 or H9N2 avian influenza viruses in 11 day old embryonated chicken egg. The embryos were observed daily and the virus titer was determined by HA test. There was an increase in virus titers in both simultaneous and pre-incubated groups as compared to the group inoculated with virus only. Moreover, it was observed that the higher the protease concentration, the higher the virus titer (Table 2) and chicken embryos died within 12-24 hrs post inoculation. On the contrary, *M. gallisepticum* showed no increase in virus titer except for the pre-incubation of H9N2 with 150 u/ml of *M. gallisepticum* protease.

Table 2: Simultaneous H5N1 and H9N2 inoculation in embryonated chicken eggs with different proteases concentrations.

Virus type	Proteases	Different protease concentrations (unit/ml)			Virus only
		50	100	150	
H9N2	*P. aeruginosa*	2^{7}*	2^{8}	2^{12}	2^{5}
	S. aureus	2^{6}	2^{8}	2^{12}	
	M. gallisepticum	2^{3}	2^{4}	2^{6}	
H5N1	*P. aeruginosa*	2^{6}	2^{10}	2^{12}	2^{6}
	S. aureus	2^{9}	2^{8}	2^{10}	
	M. gallisepticum	2^{3}	2^{5}	2^{7}	

*Virus titer assessed by HA test.

Table 3: Inoculation of pre-incubated viruses (H5N1 and H9N2) with different protease concentrations.

Virus type	Proteases	Different protease concentrations (unit/ml)			Virus only
		50	100	150	
H9N2	*P. aeruginosa*	2^{6}*	2^{9}	2^{10}	2^{5}
	S. aureus	2^{5}	2^{6}	2^{8}	
	M. gallisepticum	2^{5}	2^{5}	2^{9}	
H5N1	*P. aeruginosa*	2^{9}	2^{11}	2^{12}	2^{6}
	S. aureus	2^{6}	2^{8}	2^{12}	
	M. gallisepticum	2^{3}	-ve	-ve	

*Virus titer assessed by HA test.

DISCUSSION

Co-infecting bacterial pathogens are a major cause of morbidity and mortality in influenza virus infection (Mancini et al., 2016). *Staphylococcus aureus* and *Pseudomonas aeruginosa* were the most common pathogens causing co-infection with influenza virus (Klein et al., 2016). Influenza virus requires proteolytic cleavage activation of HA before infection and the process of virus activation is important determinant of viral virulence (Bosch et al., 1981; Klenk et al., 1994; Chen et al., 1998). After cleavage, the precursor protein HA0 is separated into HA1 and HA2, which is required for successful viral entry by receptor mediated endocytosis. Callan et al., (1997) demonstrated that proteases from *S. aureus* are capable of direct cleavage activation of some influenza viruses and Scheiblauer et al., (1992) reported that *Pseudomonas aeruginosa* indirectly activates HA by activating host proteases. A number of previous studies have mentioned the co-infection between influenza virus and *M. gallisepticum* (Dergham et al., 2015) but to our knowledge, there is no study about cleavage activation of influenza virus HA by *M. gallisepticum*. Microbial proteases contribute to enhancing cleavage activation of influenza viruses in birds by one or two of the following: 1) direct cleavage of HA by exogenous protease(s), 2) activation of host proteases capable of cleaving HA (e.g. plasmin, thrombin, or kallikrein) such as bacterial exotoxins and staphylokinase produced by *S. aureus* (Bottcher et al., 2013) that are able to activate eukaryotic plasminogen then activating the HA, 3) destruction of host protease inhibitors (e.g., α1-antitrypsin, C1-inactivator, and α2-macrogobulin), and 4) induction of host-mediated inflammatory responses giving rise to increased secretion or leakage of host proteases (Callan et al., 1997).

In this study, we examined the effect of the most co-infecting pathogenic bacteria (*Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Mycoplasma gallisepticum*) in promoting cleavage activation of influenza viruses HA. Especially, the effect of *M. gallisepticum*, a major poultry pathogen encoding a gene of a putative cysteine protease (CysP) similar to papain cysteine protease (C1A subfamily) on influenza virus HA cleavage activation (Cizelj et al., 2011). The pathogenic bacterial supernatant was screened for protease production on skim milk agar media containing different substrate concentrations. Well-developed clear zone was observed on 1% skim milk agar indicating that is a good media for screening of the protease as reported by Shuva et al., (2015).

The ability of bacterial supernatant to activate HA cleavage of influenza virus was assessed using embryonated chicken eggs (ECE) by detecting viral infectivity. Callan et al., (1997) reported that the allantoic fluid of eggs contains a protease that activates influenza virus allowing replication in ECE. When eggs are inoculated with uncleaved virus, the allantoic fluid proteases cleave some of these viruses sufficiently allowing infection of the ECE. In this study, inoculation of H5N1 and H9N2 viruses with different protease concentrations simultaneously and pre-incubated showed that the virus titer was increased above the basal level with both viruses as shown in table 2 and 3. But the rate of dead embryos was higher in eggs inoculated with proteases treated viruses specially H9N2 where the ECE inoculated with proteases treated H9N2 died within 24 hrs unlike ECE inoculated with non-treated H9N2 virus only died after 36 hrs and those inoculated with treated and non-treated H5N1virus died within 18-24 hrs. This could indicate that the protease in case of H9N2 virus enhanced the HA cleavage thus enhancing the infectivity, unlike H5N1 which is even enhanced by the protease, still able of utilizing the allantoic fluid proteases as mentioned by Callan et al., (1997). Thus, bacterial proteases could change the pathogenicity potential of low pathogenic H9N2 viruses.

The HAs of H5N1 highly pathogenic avian influenza viruses contain the multiple basic amino acid sequence R-X-K/R-R, making them susceptible to host intracellular proteases. The presence of these proteases in virtually all tissues contributes to the systemic spread and high virulence of these viruses in birds. In contrast, the HAs of H9N2 low pathogenic avian and other mammalian viruses are not susceptible to cleavage by these intracellular proteases and are cleaved by extracellular serine proteases (Callan et al., 1997). This was observed in our results as microbial proteases-treated viruses, either simultaneously or pre-incubated, significantly showed increased titers with H9N2 comparable to H5N1 titers. The cleavage activation of HA in H9N2 by cellular proteases was enhanced in the presence of microbial proteases in contrast to H5N1 revealing the role of bacterial proteases in HA cleavage activation of H9N2 increasing its pathogenicity. The proteases of *P. aeruginosa* and *S. aureus* are serine proteases, which strongly activate the HA cleavage resulting in high virus titers in contrast to *M. gallisepticum*, which produce cysteine protease of lower HA activation power that could have resulted in the lower virus titers detected with the viruses treated with the *M. gallisepticum* protease.

CONCLUSION

Pathogenic respiratory bacteria are considered the main cause behind the increased morbidity and mortality in influenza virus co-infection. H9N2 low pathogenic avian influenza virus infectivity was enhanced by *S. aureus* and *P. aeruginosa* possibly through increasing the HA cleavability by its own proteases either directly or indirectly. Further, *M. gallisepticum* secrets cysteine protease that could have affected HA cleavage activation but to a lower extent than the serine proteases produced by *S. aureus* and *P. aeruginosa*. Further studies are still required to understand the exact mechanism(s) by which bacterial proteases could affect virus pathogenesis, especially with low pathogenic avian influenza viruses such as H9N2 viruses that could be a potential threat for the poultry population.

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