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1 **Sensitivity of *Chlamydia suis* to cathelicidin peptides**

2

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1

2 **Abstract**

3 Nine *Chlamydia suis* isolates, obtained from pigs with conjunctivitis, were molecularly
4 characterized by *ompA* sequencing and their *in vitro* susceptibility to six cathelicidin peptides
5 (SMAP-29, BAC-7, BMAP-27, BMAP-27, BMAP-28, PG-1, LL-37) determined in cell culture.
6 SMAP-29 was the most active peptide, reducing the intracellular inclusion number by $\geq 50\%$ at a
7 concentration of 10 $\mu\text{g/ml}$ (3 μM) in six of the 9 isolates tested. Three molecularly identical isolates
8 were insensitive at a concentration as high as 80 $\mu\text{g/ml}$ (25 μM). Of the remaining cathelicidin
9 peptides tested, BAC-7 and BMAP-27 were active against six *C. suis* isolates at a concentration of
10 80 $\mu\text{g/ml}$ (25 and 26 μM , respectively). Cathelicidins LL-37 and PG-1 did not show any anti-
11 chlamydial activity at 80 $\mu\text{g/ml}$.

12

13

14 Key words: *Chlamydia suis*, cathelicidin peptides

15

16 **1. Introduction**

17

18 *Chlamydia suis* cause conjunctivitis, pneumonia, enteritis, genital tract diseases and a high
19 incidence of apparently asymptomatic infections in swine; the high incidence of *C. suis* in enteric
20 porcine specimens indicates that it may be endemic in pig herds (Longbottom, 2004).

21 Several studies have reported that granular protein extracts from mammalian polymorphonuclear
22 leukocytes inactivate *Chlamydia* spp. (Register et al., 1987): these antimicrobial peptides include
23 defensins and cathelicidins. The cathelicidin peptides are heterogeneous in size and sequence and
24 exhibit marked structural diversity (Gennaro and Zanetti, 2000). Previous studies have investigated
25 the antimicrobial activity (Frank et al., 1990; Agerberth et al., 1995; Skerlavaj et al., 1999) of
26 cathelicidin peptides against bacteria, fungi and protozoa. Recently, cathelicidin peptides have been

1 tested against *Chlamydia* isolated from humans (Yasin et al. 1996a; Yasin et al., 1996b; Turner et
2 al., 1998; Donati et al., 2005) as well as from various animal species (Donati et al., 2005), but not
3 from pigs. The susceptibility of *Chlamydia* to cathelicidin peptides differs with *C. trachomatis*
4 showing far more sensitive to antimicrobial peptides than chlamydiae isolated from animals (Donati
5 et al., 2005). In the present study, we investigated the *in vitro* activity of six cathelicidin peptides
6 against nine *C. suis* isolates.

7

8 **2. Materials and methods**

9

10 *2.1. Origin of samples*

11

12 Nine *C. suis* isolates (MS04 and MS06 1–8) were collected from conjunctival swab specimens
13 obtained from pigs with conjunctivitis from different herds in Northern Italy .

14

15 *2.2 C. suis DNA molecular analysis*

16

17 DNA of *C. suis* isolates grown in LLC-MK2 cells was extracted for molecular analysis employing a
18 commercially available kit (Tissue Kit, Qiagen, Düsserldorf, Germany) and used as a template for
19 an 1050-bp *ompA* gene fragment amplification (Sayada et al., 1995).

20 The amplicons were purified using a commercially available kit (QIAquick PCR purification kit;
21 Qiagen) and sequenced. Nucleotide sequences were compared with the same regions of the *C. suis*
22 type strain S45 available in the GenBank database using BLAST software.

23

24 *2.3 In vitro testing of cathelicidin peptides*

25

1 To determine the activity of the cathelicidin peptides, *C. suis* isolates were grown in LLC-MK2
2 cells (Donati et al., 2005) on 24-well plates with a glass coverslip at the bottom. Chlamydial
3 elementary bodies were purified using sucrose gradients (Moroni et al., 1996), resuspended in 0.2
4 M sucrose/phosphate/glutamic acid (SPG) and frozen in 0.5 ml aliquots at -70°C .
5 The six cathelicidin peptides – SMAP-29 from sheep, BAC-7, BMAP-27 and BMAP-28 from
6 cattle, PG-1 from pigs and LL-37 from humans – were chemically synthesized, purified,
7 characterized and provided as lyophilized peptides, as previously reported (Donati et al., 2005). To
8 determine the lowest peptide concentration required to achieve $\geq 50\%$ reduction in chlamydial
9 inclusions with respect to untreated controls, individual peptides were diluted two-fold with SPG,
10 from 80 to 2.5 $\mu\text{g}/\text{ml}$ in a volume of 150 μl in polypropylene tubes and added to an equal volume of
11 10^6 IFU/ml of elementary bodies in SPG medium. After incubation at room temperature for 2 h, a
12 100- μl aliquot from each sample was inoculated in triplicate into LLC-MK2 cells grown on 24-well
13 plates. After centrifugation at $800 \times g$ for 1 h at 33°C and incubation at 35°C for 48 h, the cultures
14 were fixed and stained for the presence of chlamydial inclusions by immunofluorescence with a
15 fluorescein-conjugated monoclonal antibody specific for the chlamydial LPS genus-specific
16 antigen, as previously described (Donati et al., 2002).

17

18 **3. Results and discussion**

19

20 The *ompA* amplification showed the expected products. Alignment of the deduced amino acid
21 sequences of the MOMP protein of the isolates with the same sequence of the reference *C. suis* S45
22 strain revealed an amino acid homology of 83–88%. Most of the differences were clustered in the
23 region of the variable segments (VS) I–IV of the *ompA* gene locus (Figure 1), in agreement with
24 previous data (Kaltenboeck et al., 1997; Hoelzle et al., 2000). In particular, the amino acid
25 sequences of the MS06 2, 4 and 6 isolates were identical; the sequences of MS06 3, 5 and 8 also
26 showed 100% homology. The amino acid sequences of MS06 1, 7 and MS04 differed individually.

1 The activities of the six cathelicidin peptides tested against *C. suis* isolates are shown in Table 1. Of
2 the six cathelicidin peptides tested, SMAP-29 was the most active compound against *C. suis* strains,
3 reducing the inclusion numbers by $\geq 50\%$ in six of nine isolates at a concentration of 10 $\mu\text{g/ml}$,
4 whereas BAC-7 and BMAP-27 inhibited replication of the same *C. suis* isolates at a concentration
5 of 80 $\mu\text{g/ml}$. BMAP-28, PG-1 and LL-37 had no inhibitory effect on these isolates even at a
6 concentration of 80 $\mu\text{g/ml}$. Three isolates, MS06 3, 5 and 8, molecularly identical, were not
7 sensitive to cathelicidins at a concentration as high as 80 $\mu\text{g/ml}$.

8 In a previous study (Donati et al., 2005), we comparatively analyzed the action of cathelicidin
9 peptides against several *Chlamydia* spp., including chlamydiae from men (*C. trachomatis* and *C.*
10 *pneumoniae*) and chlamydiae (*C. psittaci*, *C. pecorum*, *C. abortus*, *C. felis*) isolated from animals
11 other than pigs. *Chlamydia* showed different susceptibilities, with *C. trachomatis* showing far
12 greater sensitive to antimicrobial peptides (SMAP-29, BAC-7, BMAP-27, BMAP-28) than *C.*
13 *pneumoniae*; SMAP-29 was the most active peptide, being active against all *C. trachomatis* strains
14 tested at a concentration of 10 $\mu\text{g/ml}$. Animal chlamydiae were not sensitive; the only exception
15 being four *C. felis* isolates that were partially susceptible to BAC-7 and SMAP-29 at a
16 concentration of 80 $\mu\text{g/ml}$. In this study, six *C. suis* isolates were sensitive to SMAP-29 at a
17 concentration of 10 $\mu\text{g/ml}$, and to BAC-7 and BMAP-27 at 80 $\mu\text{g/ml}$, whereas the remaining three
18 isolates were insensitive to all cathelicidins tested. Therefore, the majority of *C. suis* isolates were
19 sensitive to cathelicidins, in particular to SMAP-29, showing a sensitivity approaching that of
20 human *C. trachomatis* isolates (Donati et al., 2005). This observation is not unexpected, since *C.*
21 *suis* strains were previously referred to as *C. trachomatis*. Even if porcine strains are genetically
22 different to human strains, a close relationship between *C. suis* and *C. trachomatis* is indicated by
23 the *ompA* DNA sequence homology (Kaltenboeck et al., 1997), together with morphology and other
24 characteristics, such as the production of glycogen by *C. suis* in cell culture (Rogers et al., 1996). It
25 has been also reported that several *C. suis* strains possess a plasmid (Everett, 2000). This close
26 biological relationship is further confirmed by the results of this study in relation to sensitivity

1 against cathelicidin antimicrobial peptides. Finally, it is of interest to note that the three *C. suis*
2 isolates insensitive to cathelicidins were molecularly identical compared to the sensitive isolates,
3 which showed nucleotide differences, mostly clustered in the region of the VS I to IV of the *ompA*
4 gene locus. This observation deserve further study to evaluate whether these genetic differences
5 could be correlated with insensitivity to cathelicidin peptides.

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8
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10

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9 **Legend to Figures**

10 Fig. 1. Alignment of deduced amino acid sequence of *ompA* PCR products from nine *C. suis*
11 isolates with reference strain *C. suis* S45.
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      10      20      30      40      50
S45  ALPVGNP AEP SLM IDG ILWEGFGGDP CDP CTTWCDAVS LRLGGYGDYVFD
MS04  .....
5_MS06 .....
8_MS06 .....
3_MS06 .....
4_MS06 .....
2_MS06 .....
6_MS06 .....
7_MS06 .....
1_MS06 .....

      60      80      90     100
      VS I
S45  RVLKTDVAKQFDMGDTPSNTNGNT--TSPTQLTARDNPAYGKHFQDAEMF
MS04  .....E.E..KA.EPTDQDS.V.A.LTPI..P.....
5_MS06 .....I.E..E.GT..AN-----A...TKS.E.....
8_MS06 .....I.E..E.GT..AN-----A...TKS.E.....
3_MS06 .....I.E..E.GT..AN-----A...TKS.E.....
4_MS06 .....D.E..KLYTADNA-----TS.TRETTS.V.....
2_MS06 .....D.E..KLYTADNA-----TS.TRETTS.V.....
6_MS06 .....D.E..KLYTADNA-----TS.TRETTS.V.....
7_MS06 .....D.E..AA.TKQDTOKLQ.A.LTPIS.E.....
1_MS06 .....E.E..AA.TKQDTTKLQND.KTP.P.P.....

      110     120     130     140     150
      VS II
S45  TNAAFMALNIWDRFDVFC TLGATNGYLKGN SASFNLVGLFGAN--ETTAA
MS04  .....A.....DPTQSSQV.
5_MS06 .....A.....DQ.TI
8_MS06 .....A.....DQ.TI
3_MS06 .....A.....DQ.TI
4_MS06 .....A.....LT--AQ.VG
2_MS06 .....A.....LT--AQ.VG
6_MS06 .....A.....LT--AQ.VG
7_MS06 .....A.....DPTQAAQV.
1_MS06 .....VSNQDS--I

      160     170     180     190     200
S45  QATAVPNVSLSQSVVELYTD TAFAWSVGARAAALWECGCATLGASFQYAQS
MS04  TSKNL.....A.I.....
5_MS06 PEA.....A.A.....
8_MS06 PEA.....A.A.....
3_MS06 PEA.....A.A.....
4_MS06 -.DL..AT.T.A.....
2_MS06 -.DL..AT.T.A.....
6_MS06 -.DL..AT.T.A.....
7_MS06 TTKNL.....A.I.....
1_MS06 EKDVL.....A.....

      210     220     230     240     250
      VS III
S45  KPKVEELNVL CNAAEFTINKPQGYVGVEFP LDKAGTTAATGTKDASIDY
MS04  .....K.....EIT..D.....
5_MS06 .....Q.....PKT..SQ..DV.....
8_MS06 .....Q.....PKT..SQ..DV.....
3_MS06 .....Q.....PKT..SQ..DV.....
4_MS06 .....Q.....QA..PET..SN..DV.....
2_MS06 .....Q.....QA..PET..SN..DV.....
6_MS06 .....Q.....QA..PET..SN..DV.....
7_MS06 .....K.....QA..EFT..D.....
1_MS06 .....F..KD..E.T..D.....

      260     270     280     290     300
      VS IV
S45  HEWQASLALSYRLNMF TPIYGKWSRASFDADTIRIAQPKLATAVFDVTT
MS04  .....
5_MS06 .....Q.IYE...
8_MS06 .....Q.IYE...
3_MS06 .....Q.IYE...
4_MS06 .....A.SPIGQT..
2_MS06 .....A.SPIGQT..
6_MS06 .....A.SPIGQT..
7_MS06 .....GLMPTQF.MLS--TRL SLSRRHH
1_MS06 .....AL..THSHRSA-N-----

      310     320     330
S45  LNPTIAGAGKVEDKGSAGELGDTMQIVSMQINKMK
MS04  .....
5_MS06 .....Q.....P.T.....
8_MS06 .....Q.....P.T.....
3_MS06 .....Q.....P.T.....
4_MS06 .....S.K..-IDAND.....
2_MS06 .....S.K..-IDAND.....
6_MS06 .....S.K..-IDAND.....
7_MS06 I.....
1_MS06 I.....

```

1 Table 1. Activity of cathelicidin peptides against nine *Chlamydia suis* isolates.

2

3

Peptide concentration [$\mu\text{g/ml}$ (μM)] reducing chlamydial inclusion by $\geq 50\%$

4

Strains -----

5

SMAP-29

BAC-7

BMAP-27

BMAP-28

PG-1

LL-37

MS04	10 (3)	80 (19)	80 (>25)	>80 (>26)	>80 (>37)	>80 (>18)
5MS06	>80 (>25)	>80 (>19)	>80 (>25)	>80 (>26)	>80 (>37)	>80 (>18)
8MS06	10 (3)	>80 (19)	>80 (25)	>80 (>26)	>80 (>37)	>80 (>18)
3MS06	>80 (>25)	>80 (>19)	>80 (>25)	>80 (>26)	>80 (>37)	>80 (>18)
4MS06	10 (3)	80 (19)	80 (25)	>80 (>26)	>80 (>37)	>80 (>18)
2MS06	10 (3)	80 (19)	80 (25)	>80 (>26)	>80 (>37)	>80 (>18)
6MS06	10 (3)	80 (19)	80 (25)	>80 (>26)	>80 (>37)	>80 (>18)
7MS06	10 (3)	80 (19)	80 (25)	>80 (>26)	>80 (>37)	>80 (>18)
1MS06	10 (3)	80 (19)	80 (25)	>80 (>26)	>80 (>37)	>80 (>18)

6

7

8

9