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Original article

Transmission and pathogenicity of encephalomyocarditis virus (EMCV) among rats

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Abstract – Due to the probable role played by rodents as a reservoir for the transmission of the EMC virus to pigs, the experiment reported here was performed in order to assess the transmission rate of EMCV within a rat population. Twenty-five eight-week-old Wistar rats housed in individual plastic cages were experimentally infected either with a Greek myocardial EMCV strain (5 rats with a 0.2×10^6 $TCID_{50}$ dose per rat and 10 rats with a $0.5 \times 10^{4.5}$ $TCID_{50}$ dose per rat, oronasally) or a Belgian myocardial EMCV strain (10 rats with a $0.5 \times 10^{4.5}$ $TCID_{50}$ dose per rat, oronasally). Two to five days later, each inoculated rat was moved to a new clean cage and coupled with a contact rat to compare the pathogenicity of the two strains and to estimate the basic reproduction ratio R_0, indicating the level of EMCV transmission. During the experiments, faecal virus excretion was measured as well as the serological response against EMCV. After euthanasia, virus isolation was attempted from different rat tissues. Neither strains produced mortality, nor clinical signs and only low titres of neutralising antibodies were found. All contact rats, however, were infected and the virus was isolated from their faeces and from various tissues. Both 10-pair experiments revealed a point estimate for the R_0 of ∞ (95%-CI for both the Greek and Belgian EMCV strains = $4.48 - \infty$), as did the 5-pair experiment with a higher dose of the Greek strain (95%-CI = $1.83 - \infty$). Combining the results from the two 10-pair experiments resulted in an estimate for R_0 of ∞ (95%-CI: $9.87 - \infty$). These results indicate that the EMC virus can spread very easily within a rat population by horizontal rat-to-rat transmission ($R_0 >> 1$).

encephalomyocarditis virus / rats / pathogenicity / transmission / R_0

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1. INTRODUCTION

Encephalomyocarditis virus (EMCV) is a member of the genus *Cardiovirus* of the family Picornaviridae, with a worldwide distribution [13]. Rodents are considered as the natural host of EMCV [1].

In domestic pigs, EMCV has been recognised either as a cause of mortality in young pigs, due to acute myocarditis, or of reproductive failure in sows [27]. The myocardial form has been reported in young pigs in Greece [18, 22], Italy [14, 24], Cyprus [18] and Belgium [17, 18]. The reproductive form, characterised by abortions, still- or weakborn piglets and mummification, has been reported in Belgium only [15]. These apparently conflicting reports suggest that EMCV strains may vary in pathogenicity and tissue tropism. Each form of the disease in pigs seems to be restricted to certain geographical areas, probably due to viral strains originating from local rodent populations. With respect to EMCV infections in pigs, at present time two routes of infection are suggested for the introduction and/or subsequent spread of the virus within a pig farm. At first, pigs might get infected by the ingestion of substances (e.g. faeces or carcasses) of infected rodents [1, 20, 23]. The second route is horizontal pig-to-pig transmission during the short period of viraemia [4, 17] or after reactivation of EMCV persistence [5]. From a recent study by Maurice et al. [21], in which the EMCV transmission from pig-to-pig contact was experimentally quantified, it can be concluded that the spread of EMCV between pigs in most cases will be limited. The high seroprevalence levels found in the field and the observed clinical infections in separated pens and compartments of affected pig houses might therefore point to an additional spreading mechanism, for example via rodents. Although it is known that rats can be infected with the EMC virus [2], only little is known about the spread of the virus within the rat population. More insight into the transmission of EMCV among rats might enlighten their role as a possible transmitter or a potential reservoir for the EMC virus and stress the need for an effective rodent control programme at the farm level. A commonly used measure of the transmission of infectious agents is the basic reproduction ratio (R_0), which is defined as the mean number of new infections that one typical infectious individual causes in a totally susceptible population [3, 6]. R_0 was used to quantify the transmission of the EMC virus among rats.

The purpose of the present experimental work was (a) to study the pathogenicity of two EMCV strains for rats and (b) to experimentally quantify the horizontal rat-to-rat transmission of EMCV. An R_0-value above one would indicate that the EMCV virus is able to spread within the rat population by horizontal rat-to-rat transmission and therefore the hypothesis H_0: $R_0 < 1$ was tested.

2. MATERIALS AND METHODS

2.1. Animals and experimental design

Fifty eight-week-old Wistar rats were obtained from the Theagenion Anticancer Institute of Thessaloniki, Greece. The rats were free of EMCV as assessed by serological and virological examinations before inoculation.

Three experiments were conducted successively, studying the pathogenicity and transmissibility of two EMC virus isolates. In each experiment, the rats were randomly assigned into two groups and each rat was housed in an individual plastic cage (26 × 20 × 14 cm). In experiment A, ten rats were infected oronasally with a $0.5 \times 10^{4.5}$ $TCID_{50}$ dose per rat of the Greek strain 424/90. Two days after infection each rat was transferred to a new clean cage, together with an uninfected contact rat. Infected and contact rats were euthanized 18 to 59 days post infection (p.i.) (Tab. I). In experiment B, ten rats were infected oronasally with a $0.5 \times 10^{4.5}$ $TCID_{50}$ dose per rat of the Belgian

Table I. Virus isolation from tissue samples in rats experimentally infected oronasally with 0.5 mL × $10^{4.5}$ $TCID_{50}$ of the EMC virus strain G424/90 (Exp. A).

Pair No.	Rat No.	Rat status[a]	Day of introduction	Killed (days p.i. or p.c.)	VNT[b]	Faeces[c]	Heart	Spleen	Lung	Liver	Brain	Kidney	Pancreas	Peyer's patches	Thymus
1	31	i		59	64	2–23	–[e]	–	–	–	–	–	–	+	+
	32	c	2	57	32	6–28	–	–	–	–	–	–	–	+	+
2	33	i		22	32	2–22	+[d]	+	+	–	–	–	+	+	+
	34	c	2	20	16	5–19	+	+	+	+	–	+	+	+	+
3	35	i		21	16	2–21	+	+	+	+	–	–	+	+	+
	36	c	2	19	4	9–19	+	+	+	+	–	+	+	+	+
4	37	i		58	32	2–24	–	–	–	–	–	–	–	+	+
	38	c	2	56	8	6–27	–	–	–	–	–	–	–	+	+
5	39	i		22	16	3–22	+	+	+	+	–	–	+	+	+
	40	c	2	20	8	4–20	+	+	+	+	–	–	+	+	+
6	41	i		20	8	2–20	+	+	+	+	–	–	+	+	+
	42	c	2	18	4	3–18	+	+	+	+	–	+	+	+	+
7	43	i		20	16	2–20	+	+	+	+	–	–	+	+	+
	44	c	2	18	8	7–18	+	+	+	+	–	+	+	+	+
8	45	i		21	16	2–21	+	+	+	+	–	–	+	+	+
	46	c	2	19	8	4–19	+	+	+	+	–	–	+	+	+
9	47	i		58	32	3–28	–	–	–	–	–	–	–	+	+
	48	c	2	56	32	7–29	–	–	–	–	–	–	–	+	+
10	49	i		59	64	2–25	–	–	–	–	–	–	–	+	+
	50	c	2	57	16	6–29	–	–	–	–	–	–	–	+	+

[a] i: inoculated rat, c: contact rat.
[b] Serum neutralisation titers on day of death.
[c] Time-span of virus isolation (days p.i. or p.c.).
[d] +, virus detected.
[e] –, virus not detected.

strain B275/95. Two days after infection each rat was transferred to a new clean cage, together with an uninfected contact rat. Infected and contact rats were euthanized 11 to 62 days p.i. (Tab. II). In experiment C, five rats were infected oronasally with a 0.2×10^6 $TCID_{50}$ dose per each rat of the Greek strain 424/90. To quantify the rat-to-rat transmission in experiment C, each rat was transferred to a new clean cage together with an uninfected contact control rat, at predetermined (2 to 5) days after infection. Infected rats were euthanized 3 to 7 days p.i. Contact rats were euthanized 20 to 23 days post contact (p.c.) (Tab. III).

In the current study, a contact rat was considered infected when the virus could be isolated from the faeces. After euthanasia, contact infection was confirmed by virus isolation from various tissues.

In all experiments, blood samples were taken from each rat before inoculation and on the day of death. Fresh faeces were collected before inoculation and from 2 to 32 and on 58 and 59 days p.i. (experiment A), from 2 to 32 and on 43 and 62 days p.i. (experiment B) and from 2 to 23 days p.i. (experiment C), except for those rats that were killed earlier. After euthanasia, necropsy was performed and samples from the brain, thymus, heart, lung, liver, spleen, kidney, pancreas and Peyer's patches were collected for virus isolation.

2.2. Viruses

Two EMC virus strains were used. Strain 424/90 was isolated in Greece, in 1990, from the myocardium of a three-month-old pig in a breeding farm with the typical myocardial form of EMCV [4]. Strain B279/95 was isolated during the first outbreak of myocardial disease due to EMCV in fatteners in Belgium in August 1995 [17]. Both were isolated and passaged on baby hamster kidney (BHK-21) cells. For the preparation of the viral inocula of the Greek strain, a 4th passage was performed on the same cell batch. The virus infectivity of the stock was 10^6 $TCID_{50}$/mL. In addition, for the preparation of the viral inocula of the Belgian strain the first passage was used. The virus infectivity of the stock was $10^{4.5}$ $TCID_{50}$/mL. Infected cell culture fluids were centrifuged for 5 min at 2 000 *g* to remove cellular debris, mixed 1:1 with sterile glycerol and stored at –20 °C.

The identification of the viruses was performed by neutralisation with a specific EMCV antiserum, electron microscopy, RNA-analysis and RT-PCR [16]. African and classical swine fever viruses, parvovirus, Aujeszky's disease virus, swine vesicular disease virus and porcine respiratory and reproductive virus were not detected in the inocula. Strain ATTC 129B was used for serological analysis.

2.3. Serological assay

A virus neutralisation test (VNT) was performed. Two-fold dilutions of serum were made in minimum essential medium (MEM) in 96-well flat-bottomed microtitration plates (Nunc, Denmark). One hundred $TCID_{50}$ of EMCV was added in equal volume. The plates were incubated at 37 °C in a 5% CO_2 atmosphere for 1 h before BHK-21 cells were added. The results were usually read after a 48-h incubation. The titres were expressed as the initial dilution of the sera at the 50% end point according to the method of Kärber [11]. The sera were considered positive if the titre was equal to or higher than 1/32 [19].

2.4. Virus isolation

Virus isolation was attempted from all rats as previously described [22]. In short, tissue supernatants were incubated on BHK-21 cell monolayers. The faeces were diluted 1/10 in MEM with the addition of 600 mg/L sulfadoxin, 120 mg/L trimethoprim and 500 000 IU/L of penicillin. After centrifugation at 3 000 *g* for 10 min, the supernatants were processed as for tissue homogenates. The samples that showed a cytopathic effect were submitted to a neutralisation test, using specific EMCV-antiserum to

Table II. Virus isolation from tissue samples in rats experimentally infected oronasally with 0.5 mL × $10^{4.5}$ $TCID_{50}$ of the EMC virus strain B279/95 (Exp. B).

Pair No.	Rat No.	Rat status[a]	Day of introduction	Killed (days p.i. or p.c.)	VNT[b]	Faeces[c]	Heart	Spleen	Lung	Liver	Brain	Kidney	Pancreas	Peyer's patches	Thymus
1	11	i		19	32	2–19	+[d]	+	+	–[e]	–	–	+	+	–
	12	c	2	17	16	9–15	+	+	+	+	–	+	+	+	+
2	13	i		15	16	2–15	+	+	+	–	–	–	+	+	+
	14	c	2	13	8	9–13	+	+	+	+	+	+	+	+	+
3	15	i		62	64	2–24	–	–	–	–	–	–	–	+	–
	16	c	2	60	32	15–28	–	–	–	–	–	–	–	+	+
4	17	i		13	< 2	2–13	+	+	+	+	–	+	+	+	+
	18	c	2	11	< 2	2–11	+	+	+	+	+	+	+	+	+
5	19	i		62	16	4–26	–	–	–	–	–	–	–	+	+
	20	c	2	60	8	17–28	–	–	–	–	–	–	–	+	+
6	21	i		15	8	2–15	+	+	+	+	–	+	+	+	–
	22	c	2	13	< 2	6–11	+	+	+	+	–	+	+	+	+
7	23	i		62	16	4–26	–	–	–	–	–	–	–	+	+
	24	c	2	60	8	17–28	–	–	–	–	–	–	–	+	+
8	25	i		62	16	2–22	–	–	–	–	–	–	–	+	–
	26	c	2	60	< 2	24–28	–	–	–	–	–	–	–	+	+
9	27	i		19	16	4–19	+	+	+	–	–	+	+	+	+
	28	c	2	17	4	9–17	+	+	+	+	–	+	+	+	+
10	29	i		62	16	4–19	–	–	–	–	–	–	–	+	–
	30	c	2	60	8	20–28	–	–	–	–	–	–	–	+	+

[a] i: inoculated rat, c: contact rat.
[b] Serum neutralisation titers on day of death.
[c] Time-span of virus isolation (days p.i. or p.c.).
[d] +, virus detected.
[e] –, virus not detected.

Table III. Virus isolation from tissue samples in rats experimentally infected oronasally with 0.2 mL × 10^6 $TCID_{50}$ of the EMC virus strain G424/90 (Exp. C).

Pair No.	Rat No.	Rat status[a]	Day of introduction	Killed (days p.i. or p.c.)	VNT[b]	Faeces[c]	Heart	Spleen	Lung	Liver	Brain	Kidney	Pancreas	Peyer's patches	Thymus
1	1	i		3	< 2	3	+[d]	+	+	–[e]	–	–	–	+	+
	2	c	2	20	< 2	9–16	+	+	+	–	–	–	+	+	+
2	3	i		4	< 2	2–4	+	+	+	–	–	–	+	+	+
	4	c	2	20	12	16	+	+	+	–	–	–	+	+	+
3	5	i		5	4	3–5	+	+	+	–	–	–	+	+	+
	6	c	3	21	< 2	9–20	+	+	+	–	–	–	+	+	+
4	7	i		6	4	2–6	+	+	+	+	+	+	+	+	+
	8	c	4	22	< 2	16–20	+	+	+	–	–	–	+	+	+
5	9	i		7	8	3–7	+	+	+	+	+	+	+	+	+
	10	c	5	23	< 2	16–20	+	+	+	–	–	–	+	+	+

[a] i: inoculated rat, c: contact rat.
[b] Serum neutralisation titers on day of death.
[c] Time-span of virus isolation (days p.i. or p.c.).
[d] +, virus detected.
[e] –, virus not detected.

identify the isolate. Three blind passages of 3 days each were made of the negative samples.

2.5. Statistical analysis

The performed transmission experiments were analysed by means of the "general epidemic model" or SIR-model (Susceptible-Infectious-Removed) [7]. The probability distribution of the outcome of a transmission experiment, which is the total number of contact infections (final size), can be described in terms of the transmission parameter R_0 using an algorithm given by De Jong and Kimman [8]. In the case of a one-to-one or pairwise transmission experiment, the outcome of the infection process is a binary variable: contact infection occurs or not. Therefore, the total number of observed contact infections in *n* independent replications of a pairwise transmission experiment is binomially distributed. The maximum likelihood estimator (MLE) for the probability of infection is then simply given by the observed proportion of contact infections. If this information is combined with the formula for the final size algorithm, the MLE for R_0 can be described and calculated from the number of contact infections and the number of repetitions of the experiments [21, 26].

In this study, the experiments A and B can both be considered pairwise experiments with 10 repetitions, while experiment C consisted of 5 pairwise repetitions. The boundaries of the 95% confidence interval for R_0 were calculated, together with the *p*-values to test the null-hypothesis H_0: $R_0 \leq 1$ [21]. The null hypothesis was rejected when the probability was less than 0.05.

3. RESULTS

3.1. Clinical signs

None of the inoculated or contact infected rats showed any clinical signs nor died.

3.2. Serological assay

No neutralising antibodies were detected in any of the rats before inoculation or contact. However, neutralising antibodies in low titres were detected in inoculated and contact infected rats. However the titres reached the cut-off value only in few rats of experiment A and B and in no rat in experiment C (Tabs. I, II and III).

3.3. Macroscopic lesions

No macroscopical lesions were observed in any organs of inoculated and contact infected rats.

3.4. Virus isolation

EMCV was isolated from the faeces of both inoculated and contact rats between days 2 and 29 in experiments A and B, and 2 and 20 in experiment C. In all experiments, EMCV was only isolated from the thymus and Peyer's patches from rats killed late post-infection (57–62 days post inoculation or contact), whilst EMCV was also isolated from several other tissues in rats killed sooner after infection (3–23 days) (Tabs. I, II and III).

3.5. Quantification of EMCV transmission by means of the R_0-value

A first estimate of the value of R_0 for the Greek EMC virus strain (G424/90) was obtained from experiment A. The R_0 was estimated to be ∞ (95%-CI = 4.48 – ∞) from the observed final size of 10 (total number of contact infections in the 10 pairwise repetitions) and the null-hypothesis $R_0 < 1$ could be rejected ($p = 0.000$).

The data of experiment B with the Belgian EMCV strain (B279/95) resulted in an R_0 value of infinity (∞) (95%-CI = 4.48 – ∞), which also resulted in the rejection of the null-hypothesis $R_0 < 1$ ($p = 0.000$). When the results for both the Belgian and Greek EMCV strains (dose $10^{4.5}$) were

pooled, the point estimate for R_0 did not change (∞), but the lower limit of the confidence interval was raised from 4.48 to 9.87 (95%-CI: 9.87 – ∞).

Also in the 5-pair experiment with the Greek strain (high dose, 10^6 $TCID_{50}$) all contact rats were infected, again resulting in a point estimate for R_0 of infinity (95%-CI: 1.83 – ∞) and again the null-hypothesis ($R_0 < 1$) was strongly rejected ($p = 0.004$).

4. DISCUSSION

4.1. Pathogenicity of EMCV among rats

In this experimental work, rats were infected with two different myocardial EMCV strains.

After experimental infection with either of the strains, none of the infected rats showed any clinical signs nor died. The inapparent infection of the rats was in agreement with Findlay and Howard [9], but was in contrast with Kilham et al. [12] who described paralysis and death after the experimental infection of albino rats with EMCV. It was confirmed that rats survive infective EMC doses that would be lethal for piglets and mice, and they infect contact rats. Transmission from rat-to-rat was slow, slower than in pigs [4]. EMCV was isolated from the faeces of both inoculated and contact rats between days 2 and 29 in experiments A and B, and 2 and 20 in experiment C. Infected rats seem to excrete the EMC virus in faeces for a longer period than piglets do [4]. Taking into account the resistance of all naked viruses in the environment, the possibility of transmission from rat-to-rat and rat-to-pig during this period is evident.

Neutralising antibodies were detectable in both inoculated and contact rats independently of the infectious dose. However, the titres were low and delayed after infection. This may partly account for the long viral excretion. In experiments A and B, the titre reached the diagnostic cut-off level of 1/32 [19] in only ten rats. On the contrary, all rats in experiment C, which were infected with a higher dose but killed earlier, had titres lower than the diagnostic cut-off.

Following euthanasia, no macroscopical lesions were observed in any organs of any of the rats.

The virus was isolated from several tissues of inoculated and contact infected rats in all experiments independently of the infectious dose. It was most frequently isolated from Peyer's patches and the thymus, and less frequently from other tissues. The number of positive tissues decreased with time. It should be noticed that in rats killed late post-infection, EMCV was isolated from the thymus and Peyer's patches only. The presence of the virus in the lymphoid tissues was in agreement with our previous work on pigs [5], that the macrophages of these tissues may indicate the sites of viral persistence and routes of viral shedding. In fact, the presence of virus in the Peyer's patches of most rats, in all experiments, indicates that this tissue represents a site of viral persistence after oral infection.

4.2. Transmission of EMCV among rats

The second target of this experimental work was to quantify the level of EMCV transmission between rats, as measured by the reproduction ratio R_0. This parameter has an important threshold; if $R_0 < 1$, only minor outbreaks will occur and an infection will die out, but if $R_0 \geq 1$, large outbreaks are also possible [7]. Since rats or rodents are often suggested as the potential reservoir for the EMC virus on pig farms [2, 25], information on the EMC virus transmission among rats could be used to ground this hypothesis. In many EMCV outbreaks on pig farms, plagues of rats and mice have been reported [2, 17, 23] and in some of these outbreaks rodents were also tested and found positive for EMCV on virus isolation from organs and/or intestines or faeces [2, 10, 17]. In this study, a

rat was considered infected when the EMC virus could be isolated from its faeces and contact infection was confirmed by virus re-isolation from various tissues. Based on the data from the current experiments A, B ($p = 0.000$) and C ($p = 0.004$), the hypothesis $R_0 \leq 1$ was strongly rejected for both virus strains, indicating that each infected rat would at least infect one other rat in a totally susceptible population [3]. This implies that EMCV can persist in the rat population by rat-to-rat virus transmission alone, which makes the rat population a potential reservoir for EMCV on commercial pig farms.

4.3. Relation of EMCV outbreaks on pig farms

Currently suggested transmission routes from rats to pigs are the following: (a) ingestion of infected faeces from rodents or (b) ingestion of infected rodent carcasses [1, 20, 23].

The high R_0, their survival to infection and the long period with virus excretion in the faeces could make rats a potential threat to pigs on a pig farm. Recent findings from a case study in Belgium by Kluivers et al. (unpublished data) showed that infection among pigs was widespread throughout the affected compartment. Findings from Maurice et al. [21], however, indicate that the pig-to-pig transmission in most cases will be limited (R_0 close to 1), suggesting that another species such as rats could be involved in the epidemiology of EMCV on pig farms.

Additional research is needed to elaborate the rat hypothesis and to test other existing theories. Seaman et al. [23] has suggested, for example, a role for mice in the infection chain for EMCV, an idea that might be supported by the finding of mice as a risk factor for clinical EMC as found by Maurice et al. [21].

From the current study, we conclude that both EMCV strains are transmitted very effectively among rats under experimental conditions. This might imply that they could play an important role in the epidemiology of EMCV infections on pig farms by either serving as a reservoir host or as a transmitter of the virus to the pigs. More insight is needed, however, in the contact structure within the rat population and their behaviour on pig farms.

Experiments between rats and pigs, with special emphasis on the infectious dose from rats to pigs, could provide useful information on the transmission of EMCV between these species.

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